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Effects of drought and nutrients on growth, morphology and wood anatomy of two genotypes of *Salix*

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ABSTRACT

Due to a growing global population and an increasing welfare in combination with limited resources of fossil fuels and environmental problems may bioenergy be one of the alternatives for a more sustainable energy production. Among others, *Salix* is used as biological energy source. Its energy conversion is high compared to other sources and it can be managed in a short rotation period. For future energy production of *Salix* it is important to have resource efficient genotypes with high biomass production. Thus, to secure future production, it is important to breed for production and resource use efficiency traits. Objectives with this study were to evaluate how water and nutrients affect biomass production of two genotypes of *Salix* and how xylem diameter of the same genotypes is affected by drought stress. Main hypotheses with this study are that drought stressed plants have slower and lower biomass production than well-watered plants. That available nitrogen (N) increase growth, that genotype 341 has a drought tolerance than genotype 223 and that xylem vessels are narrower for drought stressed plants compared to well-watered plants. *Salix* cuttings of the genotypes 223 and 341 from a mapping population of *Salix viminalis* and the hybrid *S. vinimalis x S. schwerinii* were planted and grown in a greenhouse in Uppsala, central Sweden, with a three week long treatment period before final harvest. Greenhouse trial includes four treatments (control) 250 ml water three times a week; water and nutrients) 250 ml water three times a week and 2 ml "Blomstra" fertilizer/ l water once a week; nutrients) 250 ml water with 2 ml "Blomstra" fertilizer/ l water once a week; water shortage treatment) 250 ml water once a week). Additionally, stems of plants grown in a field trial in northern Italy for three years were collected. The stems were irradiated with X-ray emanation for creation of a 3D-picture of the stem, the cross-section of the pictures were used for counting vessels and analyzing vessel size. Both treatment and genotype affected plant growth. Genotype 341 was more affected by the treatments than genotype 223 but genotype 223 had an overall lower production. The production of the genotypes differed mainly within treatment of high water and nutrient availability and the drought treatment. The results indicate a more resource efficient production of the genotype 341 in relation to water and nutrients and also a greater drought tolerance compared to genotype 223. Highest nitrogen content was observed in fertilized plants, however in contrast to other studies soil water supply did not significantly affect N concentration in leaves. Greater vessel area was observed in irrigated plants. Stem diameter was positively correlated with both total vessel area and amounts of vessels. Amounts of vessels also tended to be negatively correlated to $\delta^{13}C$ and to standardized WUE (water use efficiency based on $\delta^{13}C$ in relation to stem diameter). For future studies a longer treatment period is recommended as well as increased number of replicates for xylem analysis. I conclude that genotype 341 has great potential for biomass production, is better suited for biomass production than genotype 223 and therefore recommended to use in future trials.

Keywords: *Salix vinimalis*, *Salix schwerinii*, biomass production, vessel, water, nutrients

POPULÄRVETENSKAPLIG SAMMANFATTNING

Salix är ett träd som kan odlas för produktion av energi. I takt med jordens ökande befolkning och i kombination med hög levnadsstandard ökar efterfrågan på energi. I framtiden tror forskare på en minskning av fossila tillgångar och därav en ökad efterfrågan på bioenergi. Idag odlas *Salix* på jordbruksmark, vilket innebär platskonkurrens för grödor som odlas som mat för människan. Trots att *Salix* är en förhållandevis tålig gröda med små behov ökar dess produktion vid god tillgång på vatten och näringsämnen. Det betyder att bra marker gynnar produktionen. Konkurrensen mellan mat och energi på jordbruksmark är en framtida fråga att lösa.

Tack vare *Salix* tidiga blomning ger träden mat åt insekter tidigt efter en hård vinter. Detta är en speciellt viktig egenskap eftersom våra pollinerande insekter tenderar att minska i antal. Det har visats att *Salix* är effektiv på att ta upp giftiga ämnen ur marken, framförallt tungmetallen kadmium. En annan betydelsefull miljöaspekt är dess effektiva omvandling av biomassa till energi. Ur *Salix* kan man få ut mer energi per insatsvara jämfört med exempelvis både vete, raps och fossila råvaror. Detta gör att det är mer energieffektivt att odla *Salix* än spannmål för energiproduktion.

Om vi med hjälp av bioenergi från *Salix* vill kunna säkerställa den framtida energiförsörjningen är det viktigt att vi redan idag påbörjar förädlingen av tåliga sorter. Sorterna måste klara ett framtida klimat. Ett klimat som förutspås vara varierat och innehålla intensiva perioder av exempelvis regn och torka. Denna studie går därför ut på att jämföra två olika sorter med varandra i ett växthusförsök, där växterna har fått olika mängd och kombination av vatten och näring under tre veckor. Detta för att se hur de två sorterna reagerar på olika typer av tillväxtförutsättningar och hur deras behov skiljer sig från varandra. Ytterligare behandlar studien hur växtens vattentransportsystem påverkas rent utseendemässigt av torka och hur detta kan påverka den totala biomassaproduktionen. Gällande analyserna för hur vattentransporten fungerar har data samlats in från *Salix* som växt i fält i Italien under tre år.

Resultat från denna studie är att en av sorterna (”341”) klarar torka bättre än den andra (”223”). Överlag producerar sorten 341 mer biomassa än sorten 223 och är därmed mer lämpad för att använda i framtida forskning. Studien bekräftar även att både vatten och näringstillförsel ökar biomassaproduktionen samt att kväveinnehållet i bladen ökar vid tillförsel av kväve. Storleken på rören som transporterar vatten i växten visade sig vara mindre när växten utsattes för torka och antal rör ökade med en växande stamdiameter. Till vidare studier rekommenderas en längre behandlingsperiod för att öka resultatens trovärdighet.

Genom fortsatta studier på olika sorters *Salix* kommer förhoppningsvis både framtidens energitillgång och jordens biologiska mångfald att gynnas. Detta i kombination med att lösa vårt framtida matbehov är en tuff och utmanande uppgift.

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3 INTRODUCTION

In the beginning of the 1970's alternative energy sources for fossil fuel became interesting due to high petroleum prices. Interest in fast growing forest increased, there among willow (*Salix* spp.), and researchers started to develop high yield genotypes (Danfors, 1992**).** Willow is a tree grown and can be used as a renewable energy resource (Swedish Board of Agriculture, 2014) and is managed as short rotation coppices (SRC). The term "short rotation forestry" is also used concerning willow (Hoffmann and Weih, 2005). SRCs are coppice with regrowth vigour, harvested every 3-5 year and grown as purpose-grown wood. Today Sweden is one of three leading countries in Europe together with Poland and the United Kingdom within growing fast growing forest, based on area (SRCPlus, 2014). Genotypes of todays' market offers about a 50 % higher yield compared to the high yield genotypes used in the 1990's (Larsson[1]).

The demand for bioenergy in Europe is today increasing, but the supply of woodchips is not high enough. Therefore an increased production is required (SRCPlus, 2014). A limited supply of fossil fuels (Lindblad, 2011) in combination with an increasing demand for bioenergy (SRCPlus, 2014) and the negative effect of fossil fuels on the climate (Uppenberg et al., 2001), indicates that some changes have to be done in the near future. Planting more fast growing forest for biomass production is an alternative. To optimize the production it is important to have good raw material.. Water and nitrogen (N) are two important parameters for production of biomass (Chaves et al., 2003) and different genotypes respond differently to deficiency and availability of these (Weih et al., 2010; Campbell et al., 2008; Glynn et al., 2004). To find efficient genotypes it is important to know how the different genotypes react to these parameters (Chaves et al., 2003) and deeper knowledge about anatomical traits of vessels will give additional understanding of drought adaptations of plants, thus water availability influence vessels (Hilarie and Graves, 1998; Kulkarni et al., 2010; Plavcová and Hacke, 2012).

The objectives of this study are to evaluate how water and N affect two genotypes of willow, differences between genotypes and interaction effects between water and N treatments. To see if one of the genotypes is better suited for drought conditions and analyze how nitrogen and water deficit affect biomass production, allocation and root production. Finally, the study investigates how the vessel size of one genotype reacts to drought compared with well-watered conditions.

[1] Stig Larsson, European Willow Breeding AB, 2015-02-04

4 BACKGROUND

4.1 A changing climate

During the last decades the climate has been changing rapidly due to human actions and due to the increased concentration of greenhouse gases in the atmosphere in particular (IPCC, 2014). The global mean temperature has increased with about 0.7 °C within the period 1906-2005 and the temperature changes in Sweden are showing the same trend. Predictions for future climate in Sweden are milder weather and higher precipitation up north and warmer weather and unchanged precipitation rates, compared to today, in the south (SMHI, 2015). The global mean surface temperature for the period 2016-2035 is predicted to increase with 0.3-0.7 °C (IPCC, 2014). When burning fossil fuel, carbon from the earth crust is released into the atmosphere, likewise is carbon released when combusting plant material; e.g. biofuels (Uppenberg et al., 2001). Differences are, among others, when combusting plant material e.g. willow the release of CO_2 is calculated as zero (Berntsson et al., 2014), due to that the emitted CO_2 when combusting equals the uptake of CO_2 of a replanted growing tree (Berntsson et al., 2014; Heller et al., 2003). Therefore, the cycle of biofuels is shorter than for fossil fuels and thereby less environmentally damaging.

The combination of limited availability of usable energy of today and plants' ability to capture sun energy is a good reason for planting short rotation forest. The fact that willow is one of the most efficient plants when transforming sun energy to plant biomass (Christersson, 2013) is worth considering for future energy production.

With efficient energy conversions willow could produce eleven times more electricity than consumed fossil energy (Heller et al., 2003). Comparing different biofuels, willow can contribute with as much as five times more energy compared with ethanol from wheat and methyl ester from rape seed (Swedish Energy Agency, 2008). The production of bioenergy needs to be productive and energy efficient if it is going to be less environment damaging compared to fossil fuels. The impact on the environment depends among type of tillage, need for additional nutrients, energy use while growing, energy content in product and usage of rest products (Swedish Board of Agriculture, 2009).

4.2 Positive environmental effects with willow production

Growth of willow can have positive effects on the environment compared to traditional crops. Willow can increase biodiversity (Baum et al., 2012) and its pollen is very important for bee and humblebee populations due to the early growth of the plants and thereby pollen production during early spring (Christersson, 2013). This is an important trait that can help recover last years' global reduction of pollinating insects that has been shown during the last years. Pollinating insects are important to most domestic and wild plants, thus gives the insects major national economic importance (Rahbek et al., 2009).

Willow also reduces soil erosion and nutrient leakage due to a lower nutrient need and its perennial growth. A lower nutrient need means less fertilizing and lower risk of eutrophication (Swedish Board of Agriculture, 2009). Willow has the capacity of cleaning waste water from nutrients and toxic elements by water uptake. The water uptake leads to transpiration and thereby less water leakage from the site. Its capacity of cleaning and removing water makes it suitable for planting around waste disposal sites (Christersson, 2013).

Willow has a high ability of accumulating cadmium (Cd) from the soil (Reimann et al., 2015) and can therefore be used as a vegetative filter (Mirck et al., 2005). It is usable that soil is cleaned from unwanted elements, e.g. heavy metals, when planting willow, but according to Gough et al. (2013) there is a risk of accumulating the heavy metals in the food chain by browsing animals. Besides interfering in the food chain heavy metals can affect plants' water balance.

4.3 Willow production in Sweden

The majority of the willow production area in Sweden is located in the south and middle parts (Skåne, Mälardalen and Västra Götaland) (Swedish Board of Agriculture, 2014), mainly due to the high frost risk up north (Dahlgren, 1992) and it covers about 10 500 hectares (Swedish Board of Agriculture, 2014). Farmers were in the beginning of the 1990's recommended to grow willow on the least nutrient rich area; due to its great capacity to take up water, low nutrient demand and long growing season (Dahlgren, 1992). This recommendation led to low yield. The recommendations are now modified and it is now recognized that willow biomass production is favored by plant available nutrients (Swedish Energy Agency, 2008; Swedish Board of Agriculture, 2013), a pH over 6 (Swedish Board of Agriculture, 2013).

In Sweden, nutrients are among the most important environmental factors that limit growth of plants and if nutrients are not limiting, water can be a limiting factor occasionally and/or locally. Though drought stress in not common in willow plantations in Sweden today (Lindroth and Båth, 1999) it might be in the future due to changing climate. Another factor is soil type, which can lead to drought stress depending on its water holding capacity. The right combination of oxygen and water in the soil determines the plants' survival. The soil needs to be well aerated and at the same contain enough plant available water (Eriksson et al., 2011). Even though willow has the capacity to survive in low water soils (Dahlgren, 1992) studies have shown differences in water tolerance between genotypes (Weih et al., 2010). The origin of the genotype can also affect the growth rate of the shoot (Wikberg and Ögren, 2004) and a study by Dudley and Galen (2007) showed that female clones showed a greater drought stress than male clones, by having a lower water potential under drought conditions.

Willow is grown on the same type of farmers land as cereal and ley, which results in competition of land (Swedish Board of Agriculture, 2009). Reasons for not using forest land are, among others, a higher risk for damages by game species and aggravated harvesting due to hilled terrain. Lack of big roads for transportation of the biomass and long distances to combusting fabrics are other reasons (Gustafsson et al., 2007). Willow is rated as an agricultural crop and can therefore not be planted on forest land, due to that forest land does not include land cultivated as agricultural usage (Swedish Forest Agency, 2014). Though, usage of arable forest land might be potential in the future, if willow production becomes successful (Christersson, 2013). The Swedish Board of Agriculture (2009) has created different scenarios for increasing willow production in Sweden. An increasing willow production, both (1) along the coast of the south part of Sweden and (2) in the south parts of Sweden (coast and inland) will lead to less national food production; cereal, oil seed rape, milk and beef. Thereby an increasing import of food and export of environmental problems.

When the national food and fodder production is on a satisfactory level, the production of alternative energy sources can increase. Growing on bigger and today non crop areas, using a more efficient growing technique and also using optimal crop rotations are three future al-

ternatives for an increasing willow production in Sweden (Swedish Board of Agriculture, 2009).

4.4 Establishment of willow

When establishing willow, it is important that the water content of the cuttings remains high (from when taking the cuttings from its original plant) (SLU, 1994). It is also important with weed control to minimize competition between crop and weed for water and nutrients. A non-successful weed control can lead to lower yield (Tahvanainen, 1999). The usage of pesticides in willow is low compared to other crops. When growing willow for 20 years the use of chemical herbicides are mainly connected to the establishment phase. Additional weed treatment is mostly mechanical. Though, there are some problems with insects and fungi in willow plantations (Swedish Board of Agriculture, 2009).

4.5 How water deficit and nitrogen affect growth and production

4.5.1 Different types of drought stress

Drought stress occurs when the water uptake is lower than the rate of transpiration (Bray, 1997). Plants' response to drought stress can be divided into two groups. Either the plants close stomata and "avoid" drought or the transpiration function is maintained but on a lower intensity (Savage et al., 1999). When closing stomata the stomatal conductance and photosynthetic activities reduces (Pezeshki, 1998) leading to lower transpiration and thereby less water losses. Reduced photosynthetic activity leads to less incorporation of carbon (Campbell et al., 2008) and thereby lower growth rate. Drought can therefore negatively affect biomass production of shoots and roots (Pezeshki, 1998; Nakai et.al, 2010; Glynn et al., 2004).

It is shown that onset of water stress can be delayed due to a decreased osmotic potential in leaves in combination with higher water and turgor potential. Caused by the increased level of soluble sugars and decreased levels of starch, that plants gains during water stress. It is also stated that drought stress reduces gas exchange in leaves but that an elevated CO_2 mitigates the stress in willow. The elevated CO_2 increased photosynthetic rate, reduced leaf conductance and increased instantaneous transpiration rate for both willow and poplar (Johnson et al., 2002).

Abscisic acid (ABA) is a plant hormone that among others facilitates drought tolerance and the hormone usually acts antagonistic to growth hormones (Campbell et al., 2008). Newly synthetized ABA respond to stomatal closure and the concentration of ABA changes in plants during water stress (Liu et al., 1999). Thus drought conditions can trigger synthesize of ABA in roots. ABA is thereafter transported through xylem vessles, to stomata where it triggers stomatal closure (Wilkinson and Davies, 2002). The reaction is fast and concentrations ABA increases rapidly in leaves under water loss. When watering after drought, a study have shown that plants with newly synthetized ABA have a longer recovery time compared to plants that have not suffered from drought conditions. The same study also showed that younger leaves where more sensitive than mature (Liu et al., 1999).

4.5.2 Drought's effect on aboveground biomass

Aboveground biomass of willow can be reduced with 35-60 % when exposed to drought (40 % soil water content of field capacity) compared to well-watered plants (75 % soil water content of field capacity) (Wikberg and Ögren, 2007), but not all studies have observed signifi-

cantly reduced growth due to drought, for all included genotypes (Van Splunder et al., 1996). Drought can lead to leaf losses (Savage et al., 1999) and it is shown that populations from regions with lower rainfall (low- rainfall species) have a lower specific leaf area (SLA) compared to populations of vegetation in higher rainfall areas (high-rainfall species) (Cunningham et al., 1999). Observations have stated that soil electrical capacitance is correlated to stem biomass, leave biomass and stem height. This makes electrical capacitance a good indicator in amount of future produced biomass (Pitre et al., 2010). It is also shown that permanent drought stress reduces growth more than temperate drought stress (Weih et al., 2010) though, according to Savege et al. (2009) a short period of drought did not affect the resprouting capacity of neither wetland specialists nor habitat generalists.

Drought stress decreases SLA (Liu and Stützel, 2003; Weih et al., 2011). SLA is the ratio of leaf area to leaf dry mass and it indicates leaf thickness (Vile et al., 2005). The lower SLA the thicker leaf and thereby a greater photosynthetic capacity due to a higher leaf protein density (Erice et al., 2009). A higher protein density means a higher N content (Campbell et al, 2008) and a higher N content have been observed in drought suffered plants (Glynn et al., 2004; Weih et al., 2011).

4.5.3 How drought and low nutrient supply affect root growth

Drought can generate a greater biomass allocation to roots than to shoots (Glynn et al., 2004; Van Splunder et al., 1996; Lambers et al., 2006), possibly due to decreased leaf growth and increased assimilation transportation to roots (Lambers et al., 2006). Leading to a lower shoot to root ratio during drought, compared to well-watered conditions (Van Splunder et al., 1996, Lambers et al., 2006). Though, observation of allocation patterns in roots show an increasing allocation to coarse roots and a decreasing allocation to fine-roots, under irrigation and fertilization. Total root biomass was therefore the greatest when plants received both water and fertilizer compared to the control and irrigation treatment (Ryan et al., 1995). Contrary, Van Splunder et al. (1996) state that it is not necessary that drought affect root dry weight. However, root length can increase with drought (Van Splunder et al., 1996; Rytter and Hansson, 1996) and drought can also generate a greater proportion of root biomass in deeper soil layers compared to shallower (Van Splunder et al., 1996). Nitrogen deficit can also affect root length, by faster growth rate. Leading to a production of thinner roots and fewer branches, compared to plants receiving plant available N (Ericsson, 1981).

4.5.4 Drought effects on vessel diameter and xylem area

The vascular transport system of trees consist two types of tissues; xylem and phloem. Phloem transports solved sugar and other organic compounds produced in the photosynthesis. The transport goes to where there the compounds are required or stored; usually to roots or sites of growth (for example leaf production). Xylem transports water and solved nutrients from the soil through the roots and to the leaves of the plant. A tree stem consists of more xylem in relation to phloem and xylem. Xylem consists of dead and living plant cells and it transports water through tracheids and vessels. Vessels are wider and longer than tracheids. Vessels are conducted of vessel elements building up vessels and the vessels range from a few centimeters to many meters. The most of the vertical water transport moves through the vessels, the open end walls of the vessel elements (perforation-plates) provide an efficient water transporting pathway. During years of growth the xylem constructs the wood while old phloem is

sloughed off and only the youngest phloem is used for sugar transport (Taiz and Zeiger, 2007; Campbell et al, 2008).

Vessel size diameter can be affected by water conditions. Studies have shown a decreased vessel diameter during drought (Kulkarni et al., 2010; Plavcová and Hacke, 2012), increased during watering (Hilarie and Graves, 1998) and increased during fertilizing (Plavcová and Hacke, 2012). Similar results shows smaller diameter during dry periods (Worbes, 1999; Sevanto et al., 2005) and wider during wet periods (Sevanto et al., 2005).

Some studies state that vessel diameter changes and that the changes over time are related to soil water content (Sevanto et al., 2005; Offenhalter et al., 2001) in the A-horizon and daily vapor deficit (Sevanto et al., 2005). Predawn xylem diameter has been observed shrinking, due to decreasing soil water content and when it started to rain, the shrining ceased and the xylem re-expanded. The same study states that daily xylem diameter changes are greater in the top of the tree than in the bottom (Offenhalter et al., 2001). Those changes may be related to the results of Moltz and Keppler (1973) who observed deformations in the phloem (92 %) and immature xylem (still living cells). Though Moltz and Keppler (1973) could not observe any deformation in the mature xylem (non-living) due to cellular water content variations, which they explain by the rigid formation of the xylem and the rigid formation makes the xylem resistant to lateral compression and keeps the plant more tolerant to water stress. Moltz and Keppler (1973) also mean that younger phloem tissues are more affected by the plant water potential and have a stronger relationship to diurnal changes in stem diameter compare to older tissue. They also state that there is no simple relationship between plant water status (e.g. xylem water potential) and stem diameter changes.

There are different methods for analyzing plant vessel diameter and total area of the vessels. Examples are with a camera lucida (Davis et al., 1999), a microscope or a Nanowood Multi-resolution X-ray CT scanner (Beyer et al., unpublished). The X-ray method is new and accurate, contributing with a deeper understanding of the genotypic differences of water use efficiency, for example due to the possibilities in formation of 3D-pictures. The phoenix v|tome|x (GE Measurement and Control, 2015, USA) is an X-ray machine and can produce both 2D- and 3D-pictures; in this study 2D pictures of the vessels were used. The analyzed object is placed in an X-ray machine and illuminated with radiation. First two-dimensional X-ray images are taken while the object rotates 360°. The images contain information about density and position of everything in the object that absorb radiation, sent from the X-ray source. The 2D images are thereafter reconstructed to 3D images (GE, 2014). The 3D images can thereafter be divided into horizontally images and e.g. showing a cross-section of a stem, done for this study.

4.5.5 Water use efficiency and $\delta^{13}C$

Plants' ability of water use efficiency refers to carbon gain per water loss and can be described in two ways: intrinsic water use efficiency (WUEi) or water use efficiency (WUE) (Lambers et al., 2006). Differences between WUE and WUEi are that WUEi is the instantaneous rate of assimilation and transpiration (Condon et al., 2002) and WUE is used in a wider concept, e.g. defined as growth of aboveground biomass per unit water consumed (Slípková and Pokorný, 2012). A third concept is standardized WUE, which is WUEi based on the carbon isotope composition ($\delta^{13}C$) in relation to stem diameter, which is closely related to total leaf area of the plant. $\delta^{13}C$ is the $^{13}C/^{12}C$ ratio related to an international standard, the lime-

stone Pee Dee belemnite (($\delta^{13}C$ (‰) = ((Rp/Rs))-1*x*1000); where Rp is the ratio in the plant and Rs is the ratio of the standard).

WUEi describes CO_2 fixation of plants in relation to its water loss (Elason et al., 2004), i.e. the rate of carbon assimilation divided by transpiration (A/T) (Condon et al., 2002). WUEi can be measured as carbon discrimination, in relative values with $\delta^{13}C$ and in absolute values with $\Delta^{13}C$ (($\Delta^{13}C$ = (Ra/Rp)-1; where Ra is the $^{13}C/^{12}C$ ratio in the atmosphere and Rp is the ratio in the plant). The ratio of ^{13}C to ^{12}C in plant tissue is less than the isotopic ratio of ^{13}C to ^{12}C in the atmosphere, because plants discriminate against ^{13}C during photosynthesis, due to their stomatal limitations and enzymatic processes. Though, measuring $\Delta^{13}C$ gives no information of which the driven variable is; carbon assimilation or transpiration (Condon et al., 2002), which should be taken in consideration before choosing between measuring A/T or $\Delta^{13}C$.

Higher WUEi can be provided by either lower stomatal conductance and/or higher photosynthetic capacity (Condon et al., 2002) and this generates in greater growth due to lower water use and/or greater biomass production compared to a lower WUE. Plants with a higher drought tolerance often have lower stomatal conductance, leading to lower photosynthesis and transpiration rates (Lamberts, 2002), plants with a higher drought tolerance will therefore increase its $\Delta^{13}C$ content.

4.5.6 Size of annual rings

Size of annual rings is dependent on water availability. Higher precipitation leads to increased cambial growth, thereby bigger rings (Worbes, 1999) and annual precipitation variation have shown to be positive correlated with radial growth of the stem, drier period generates in smaller ring widths (Walker et al, 2015).

Size of annual rings can be affected by different factors; observations have showed a relation between size of annual rings in willow and reintroduction of wolves. The reintroduction caused fewer herbivores and less browsing leading to greater growth-ring areas. Breeding for less tasty genotypes of willow is important when wanting a high biomass production, due to browsing and thereby lower growth (Beyer et al., 2007). This trait is also important for avoiding insect attacks, though Barbour (2015) stated different genotypes of willow have different levels of herbivory response.

4.5.7 How nitrogen content in leaves can be affected

Studies have stated that populations from regions with lower rainfall (low-rainfall species) have a higher N in their leaves per unit leaf area than populations in vegetation at higher rainfall (high-rainfall species) (Wright et al., 2002; Cunningham et al., 1999). Low-rainfall species have a lower concentration of internal CO_2, at a given stomatal conductance, indicating that they may have a greater photosynthetic rate than high-rainfall species. This partly explains the greater N in low-rainfall species. The lower water use makes the high N in the leaves possible (Wright et al., 2002). It is also shown that area-based leaf N content is higher, 12 % according to Glynn et al. (2004) and 16 % according to Weih et al. (2011), in drought suffered willow plants, compared to well watered plants. The higher N is acclimations to optimize N economy (Weih et al., 2011).However do plants need water to take up nutrients, due to that solved nutrient enter the plant together with soil water through roots (Karlsson et al,

1999). So, too low water availability will not increase N content in leaves due to that the N cannot be taken up.

In opposite to Wright et al. (2002) have Lauer and Boyer (1992) observed increasing internal CO_2 partial pressure during decreasing water potential and thereby stomatal closure. The pressure increased until the internal pressure was equal to the external pressure. That means the CO_2 uptake is not inhibited by stomatal closure and the pressure outside stomata generally was the factor able to decrease the internal pressure (Lauer and Boyer, 1992). So, if there is no reduction in photosynthesis, there should be no change in leaf N.

Drought is not the only factor the N in leaves depends on. For example, a higher N content in the soil increases the N in the leaves (Silvola & Ahlholm, 1992). Though, nitrogen is often considered a deficient element on cultivated land (Campbell et al., 2008) and this makes it an important nutrient when producing biomass. Because N deficit can lower growth (Campbell et al., 2008; Ericsson 1981; Taylor, 1993; Tahvanainen, 1999; Cunningham et al., 1999) and thereby reducing a potentially higher yield.

Nitrogen deficit has been shown to reduce leaf area and turn leaves yellow (Ericsson, 1981). A study by Savage et al. (2009) observed changes in pigment concentration under short term drought conditions for willow and they stated that chlorophyll content declined with drought and that some of the six species increased the xanthophyll content in leaf. The observed reduced leaf area (Ericsson, 1981) might depend on a reduced plasticity of the cell wall (Taylor, 1993).

Cunningham et al. (1999) state the same result as Silvola and Ahlholm (1992), that soil enrichment affects the nutrient content in plant. Species grown in vegetation with lower nutrient supply (low-nutrient species) contained a lower leaf N and had a lower SLA, compared to species grown in vegetation at higher nutrient supply (high-nutrient species).

Studies have proven that N fertilizing increases N in both the leaves (Jug et al., 1999) and the rest of the plant parts (Von Fircks et al., 2001), which is about the same result as Cunningham et al. (1999) and Silvola & Ahlholm (1992) stated. Allocation of N in the plant has shown to differ along the year, independent if the plants received fertilization or not. Nitrogen concentration in leaves, stems and roots declined during fall. In October/November the N started to increase in stems and roots to be stored in the perennial parts, while continuing to decrease in leaves. During spring, N in stems, cuttings and roots declined (with a more distinct decrease for fertilized plants) due to growth of new shoots. Retranslocation to roots during fall was more pronounced in plants not receiving additional fertilizing. There was a difference in time for allocation patterns for the treatments, but the pattern was the same independent fertilization or non-fertilized (Von Fircks et al., 2001).

As mentioned before, there is a relation between drought and N status in leaves (Wright et al., 2002; Glynn et al., 2004; Weih et al., 2011). Zhong et al. (2015) have studied a transcription factor in *Arabidopsis* (specifically transgenic AtTGA4 plants and wild type plants) and the outcome is that the transcription factor is induced by both nitrogen stress and drought. It was also shown that drought resistance and limited N stress was induced by overexpression of the transcription factor and that drought resistance depends on enhanced nitrate transport and assimilation. The transgenic plants had a higher tolerance to N stress and drought stressed plants had a higher N content, compared to wild type plants.

4.6 Hypotheses

Hypotheses for this study are:
Drought stressed plants have a slower and lower production of aboveground biomass and a higher N concentration per leaf area compared to well-watered plants.

- Plants of genotype 341 has greater drought tolerance than plants of genotype 223.
- Available N increases biomass production and the combination of available N and water generates in the highest biomass production for this study.
- The roots of the drought stressed plants have longer but thinner compared to well-watered plants.
- Vessel diameter of drought stressed plants decreases compared to well-watered plants.

5 MATERIAL AND METHODS

5.1 Genotypes of willow, used in this study

All plants within the same genotype has the same origin heritage, they have the same heredity (SLU, 1994). In this study two genotypes are used; 223 and 341. They both come from a F1 population from crossing the diploid female genotype L78183 (*S. viminalis*) and the diploid male hybrid cultivar "Björn" (*S. vinimalis x S. schwerinii*).

The genotype L78183 entered the market in the 1970's after joining a competition of biomass production of willow. It was collected from around Båstad, south west part of Sweden (on the coast), and the genotype turned out to have a high production rate and a relatively high frost hardiness. It has a yellowish-greenish stems with reddish-brownish spots towards the stem top. The genotype is early in the spring and cuttings should therefore not be harvested too late in the season. During the late 1980's it was used as a genotype reference for breeding and also planted in high extension during the early 1990's. Today is it replaced with newer genotypes and not available on the market (SLU, 1994; Larsson[2]).

In year 1991 Larsson[2] create the cultivar "Björn" from crossing the Siberian genotype L79069 and the genotype "Orm" (*S.* viminalis, L78195 $*$ L78101) from the company Svalöv Weibull AB. The crossing generated in very few seeds but the growing plants had a high biomass production. Its sister "Tora" have about the same production capacity but a straighter growth way and more side shoots. Neither of the siblings is flavorous by wild animals, which generates in less damaging during the establishment phase (SLU, 1994; Larsson[2]).

S. viminalis can grow well in most soils, but do not favor condensed clays and coarse-grained sands, it is also important that the soil contains enough plant available nitrogen for high biomass production (Tahvanainen, 1999). When studying willow offspring from the genotypes "L78183" and "Björn" Tsarouhas et al. (2002) observed that stem diameter correlates with stem height (r=0.82). The same study also stated that "L78183" has a higher genetic variation in growth traits than "Björn".

The used genotypes (223 and 341) has been part of a field trial in in Cavallermaggiore CN, Italy where plants of genotype 341 showed to have wider stem diameter and greater water use efficiency than plants of genotype 223 (Beyer et al., unpubl.). The differences between the genotypes are the reason for using them in this study.

[2] Stig Larsson, European Willow Breeding AB, 2015-02-02

5.2 Greenhouse experiment in Uppsla, Sweden

Stem cuttings from three year old shoots of the genotypes 223 and 341 were taken from a field south of Uppsala, Sweden and planted for growing in a greenhouse. Ten centimeter (cm) long cuttings were planted in 5.5 liters pots with a soil mixture of $^1/_3$ planting soil ("S-jord" from Hasselfors Garden) and $^2/_3$ sand (sand from Askania and "silver sand" from Sibelco). Forty cuttings of each genotype were planted and put in a greenhouse (20°C, twelve hours of artificial light per day and 60 % air humidity) for growing seven weeks before starting the treatment. Plants received water from table watering during the first seven weeks and additional water in pots when needed (to prevent the soil from drying out). Additionally each plant received 250 ml fertilized water (2 ml fertilizer "Blomstra" l^{-1} water) (Cederroth, Upplands Väsby, Sweden) one week before treatment, due to the slow growth rate. "Blomstra" is a complete fertilizer with the proportions 5:1:4:0.4 of N, P, K and Mg and 2:3 of NH_4^+ and NO_3^- (Cederroth, Upplands Väsby, Sweden) and used throughout the study.

Four sensors (two per genotype), from an EM50 data logger (Decagon Devices, Inc. (USA)), were placed in the soil and used throughout the study (approximately 7 cm from the stem) to collect data on soil moisture. Chlorophyll content was measured by SPAD-502 (Konita Minolta Sensing, Inc., Europe) at day of harvest and SPAD values are correlated to leaf N content (Weih and Rönnberg-Wästljung, 2007). Data of mean levels of N were collected for each block and treatment. Measurements were taken in the middle of the leaf and on three leaves per plant on three heights; bottom, middle and top.

The treatments were:

1) control, (watering 250 ml $plant^{-1}$ every second day) (W+N-)
2) water and nutrients, watering 250 ml $plant^{-1}$ every second day and 1.1 ml "Blomstra" $plant^{-1}$ once a week) (W+N+)
3) nutrients, (watering 250 ml and 1.1 ml "Blomstra" $plant^{-1}$ once a week) (W-N+)
4) water shortage, (watering 250 ml $plant^{-1}$ once a week) (W-N-)

Plants were seven weeks old and had an average shoot height of 27 cm when starting the treatments. Length of each shoot was measured before treatment, to divide the plants equally into treatments depending on height. Plants were placed in four randomized blocks (figure 1), in greenhouse with an air humidity of 30 %, temperature of 20°C and twelve hours of artificial light per day.

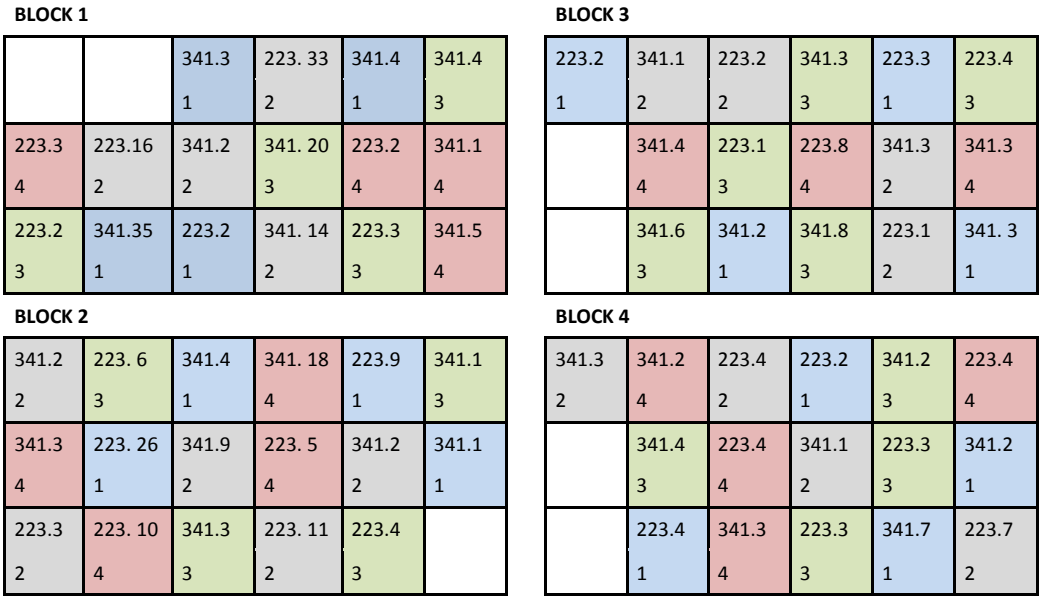

Figure 1. The design of the greenhouse experiment. Plants were placed in randomized blocks during treatment. Genotypes: 223 and 34; Individual plant number: number after the genotype on top row; Treatment: number on the bottom row (1) control (blue), 2) water and nutrients (grey), 3) nutrients (green) and 4) water shortage (red).

Baseline harvest was done when plants were eight weeks old (9th of March) (on six plants per genotype) and the final harvest was done three weeks of treatment (for all remaining plants; genotype 223: W+N-: N=7, W+N+: n=8, W-N+: n=8, W-N-: n=7; genotype 341: W+N-: n=9, W+N+: n=9, W-N+: n=9, W-N-: n=8). Harvest was done in the same way for both times. Plant height and stem diameter was measured and leaves were counted and thereafter scanned with a Ricoh Aficio MP C2051 (Ricoh Company, 2015, USA) scanner. All leaves per plant were scanned for baseline harvest and ten representative leaves per plant were scanned for final harvest. The program ImageJ-win32 (Rasband et al., 2014) was used for calculating leaf area. All plant parts were thereafter dried (in 48 hours for 70 °C) and weighted. For plants with more than one stem, the highest and widest stem were used for the statistical analysis of stem height respectively wideness, while all data were used in analysis of aboveground biomass. Soil and sand was removed from the roots under rinsing water. Three roots per plant (approximately 10 cm long) were scanned with an Epson Perfection 4990 PHOTO (Epson America, Inc., 2015, USA) scanner. Root area was analyzed with the program WinRhizo Basic, Reg & Pro 2007 (Regent Instrument Inc., 2014, Québec, QC, Canada). All roots were dried (in 48 hours for 70 °C) and thereafter weighted. Absolute and relative growth rate were calculated as equations mentioned below (T=time).

$$\text{Absolute growth rate} = \frac{\text{Final harvest} - \text{Baseline harvest}}{\text{T2} - \text{T1}}$$

$$\text{Relative growth rate} = \frac{\text{LN(Final harvest)} - \text{LN(Baseline harvest)}}{\text{T2} - \text{T1}}$$

5.3 Field experiment in Italy

Stems of genotype 223 and 341 grown in field for three years in Cavallermaggiore CN, Italy (44°42'32.4"N 7°40'40.9"E) (about 50 km south of Turin) were collected. Plants were grown under two treatments; control (receiving water by irrigating twice each vegetation period) and non-irrigated treatment. Three stems from each genotype and treatment (2 genotypes $*$ 3 replicates $*$ 2 treatments) were X-rayed with a phoenix v|tome|x (GE Measurement and Control, 2015, USA). The method is very new and a 3D-picture of an object can be produced by using the computed tomography (CT) scan (GE, 2014) and thereafter converting raw data with the GE image reconstruction software datos|x (GE Measurement and Control, 2015, USA) into TIF files. In this study only 2D-pictures were analyzed. The 2D-pictures were produced by dividing the 3D-pictures into horizontally images, showing cross-sections of the stems (figure 2). Analyses of the pictures were made with the program ImageJ-win32 (Rasband et al., 2014) obtaining xylem data and size of annual rings.

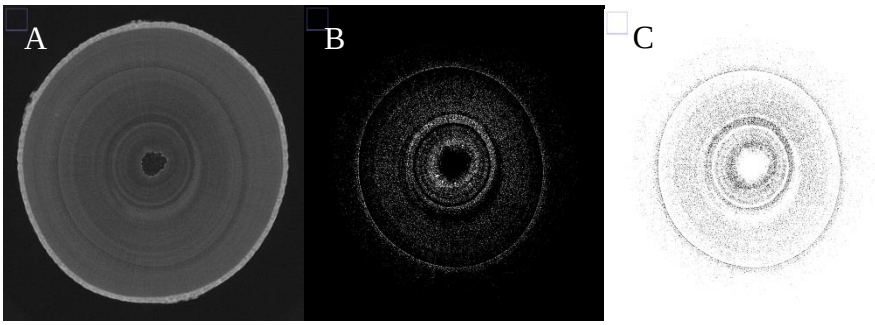

Figure 2. Cross sections picture from X-ray analysis of willow stem showing vessel as darker (A), white (B) or black (C) dots. Cross-section produced by the software datos|x (GE Measurement and Control, 2015, USA) (A). Transformed pictures of cross-sections produced with the program ImageJ-win32 (Rasband et al., 2014) (B, C).

5.4 Statistical analyzes

Normal distribution of data was tested from the residuals of the response variable that could not be explained by block, treatment, genotype or interaction effect from treatment and genotype with Shapiro-Wilk test. Homogeneity of variances (with genotype as a factor and different respons variables) was tested with Levene test. Normal distributed data and with homogeneity of variance were analyzed with multi-way ANOVA and post-hoc Tukey test. Data with non-normal distribution and/or non-homogeneity of variance were analyzed with Wilcoxon test. All statistical analyses were done with the software R (Fox, 2005).

6 RESULTS

6.1 Greenhouse experiment

6.1.1 Growth rate of different above- and belowground parameters

Absolute and relative growth rate are calculated from initial (table 1) and harvested data. Absolute and RGR of leaf area differed similar between the treatments within both genotypes (table 2; table 3; figure 3; figure 4). Fertilized plants (W+N+; W-N+) produced leaf area in a faster rate than non-fertilized plants. In conformity with growth of leaf area, the pattern of leaf biomass absolute growth rate and RGR was similar to each other (table 2; table 3; figure 5; figure 6). Within genotype 341 lead the combination of fertilization and irrigation (W+N+) to both the highest RGR and growth rate for plants of leaf area and leaf biomass, for genotype 341, comparing plants within the same genotype (table 2; table 3; figure 3; figure 4; figure 5; figure 6). Water and nutrient treated (W+N+) of genotype 223 had also the fastest absolute growth rate and RGR of leaf area, comparing plants within the genotype, though nog significantly faster than nutrient treated plants (table 2; table 3; figure 3; figure 4).

Table 1. Total amounts of biomass of genotype 223 and 341 at baseline harvest (DW per plant (without cutting biomass)). T-test table with P-values and means ± 1 SE (n=3)

	223		**341**	
	Initial biomass (g)			
	P			
Biomass for the whole plant	0.22	0.43 ± 0.09	1.66 ± 0.69	
Aboveground biomass	0.23	0.36 ± 0.07	1.43 ± 0.62	
Belowground biomass	0.14	0.06 ± 0.02	0.23 ± 0.07	
Leaf biomass	0.23	0.22 ± 0.04	0.87 ± 0.38	
Stem biomass	0.21	0.11 ± 0.02	0.56 ± 0.24	

Table 2. Comparison of absolute growth rates for leaf biomass, leaf area, stem height, stem biomass, aboveground biomass and root biomass for genotype 223 and 341 with four treatments. Computed by subtracting initial data from harvested data and divided by time period of treatment ((Final harvest- Baseline harvest)/T2-T1). Treatments: W+N- = Control, W+N+ = Water and nutrient treatment, W-N+ = Nutrient treatment, W-N- = Water shortage treatment

	Leaf		**Stem**		**Aboveground**	**Root**
Genotype; Treatment	**Area** *($cm^2\ day^{-1}$)*	**Biomass** *($g\ day^{-1}$)*	**Height** *($cm\ day^{-1}$)*	**Biomass** *($g\ day^{-1}$)*	**Biomass** *($g\ day^{-1}$)*	**Biomass** *($g\ day^{-1}$)*
223; W+N-	7.88	0.04	2.38	0.06	0.09	0.04
341; W+N-	7.95	0.04	2.25	0.06	0.10	0.07
223; W+N+	24.84	0.08	2.75	0.08	0.15	0.04
341; W+N+	30.09	0.11	3.48	0.14	0.25	0.08
223; W-N+	14.58	0.05	2.60	0.05	0.10	0.03
341; W-N+	10.02	0.03	2.66	0.06	0.09	0.04
223; W-N-	5.72	0.02	1.70	0.03	0.05	0.03
341; W-N-	1.22	0.01	2.20	0.04	0.05	0.06

Plants of genotype 223 with drought treatments (W-N-) had a higher RGR for both leaf biomass (table 3; figure 6) and leaf area (table 3; figure 4) compared to genotype 341. Addition-

ally, fertilized and drought suffered plants (W-N+) of genotype 223 had a faster leaf biomass growth rate (table 2; table 3; figure 5, figure 6) than plants of genotype 341. Furthermore plants of genotype 223 had higher RGR of leaf area (table 3; figure 6), for plants with water and nutrient treatments (W+N+) compared to genotype 341.

Table 3. Comparison of relative growth rates for leaf area, stem height and leaf, stem, aboveground and root biomass for genotype 223 and 341 with four treatments. Computed by subtracting the natural logarithm of the initial data from the natural logarithm of the harvested data and divided by time period of treatment ((LN(Final harvest)-LN(Baseline harvest))/(T2-T2)). Treatments: W+N- = Control, W+N+ = Water and nutrient treatment, W-N+ = Nutrient treatment, W-N- = Water shortage treatment

	Leaf		Stem		Aboveground	Root
Genotype; Treatment	**Area** $(cm^2\ cm^{-2}\ week^{-1})$	**Biomass** $(g\ g^{-1}\ week^{-1})^1$	**Height** $(cm\ cm^{-1}\ week^{-1})$	**Biomass** $(g\ g^{-1}\ week^{-1})$	**Biomass** $(g\ g^{-1}\ week^{-1})$	**Biomass** $(g\ g^{-1}\ week^{-1})^1$
223; W+N-	0.05	0.06	0.03	0.11	0.07	0.21
341; W+N-	0.02	0.02	0.03	0.04	0.03	0.22
223; W+N+	0.10	0.10	0.05	0.12	0.10	0.20
341; W+N+	0.05	0.06	0.05	0.08	0.07	0.24
223; W-N+	0.08	0.09	0.05	0.11	0.09	0.19
341; W-N+	0.02	0.02	0.03	0.05	0.04	0.21
223; W-N-	0.04	0.05	0.03	0.08	0.05	0.19
341; W-N-	0.00	0.01	0.02	0.04	0.03	0.23

There were some differences between the absolute and the relative growth rates of leaf area (figure 3; figure 4). Plants of genotype 223 with water and nutrient treatment (W+N+) and with water shortage treatment (W-N-) produce leaf area in a higher relative growth rate compared to genotype 341 (figure 4). The opposite proportion is shown for the absolute growth rate for plants with water and nutrient treatment (W-N+). Where plants of genotype 341 visually have faster absolute growth rate than genotype 223, though without significant differences (figure 3). On the other hand, absolute root growth rate for root biomass were significantly faster for plants of genotype 341 with water and nutrient treatment (W+N+) and water shortage treatment (W-N-) than plants of genotype 223 (figure 7).

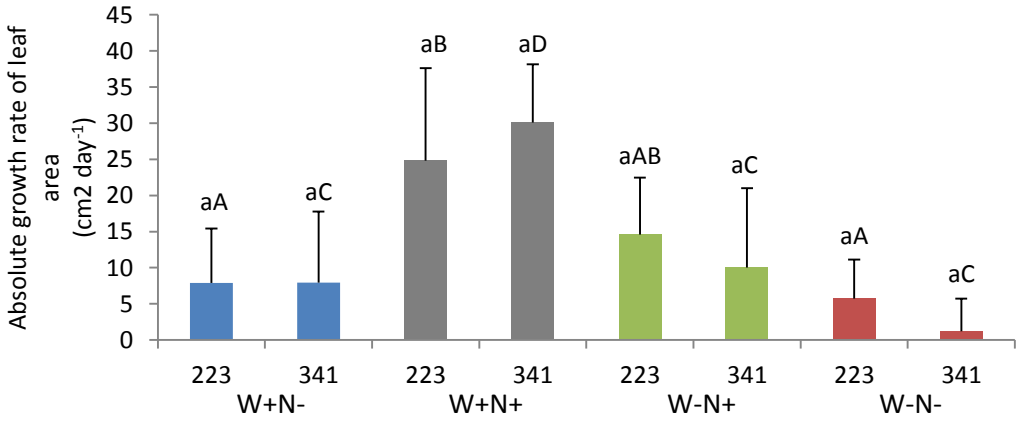

Figure 3. Absolute growth rate of mean leaf area ($cm^2\ day^{-1}$) of two willow genotypes (223 and 341), greenhouse grown in four experimental treatments. Significant differences between treatments, within genotype, are marked with different upper case letters (genotype 223: A-B; genotype 341: C-D) and significant differences between genotype, within treatment, are marked with lower case letters. Treatments: W+N- = Control (blue), W+N+ = Water and nutrient treatment (grey), W-N+ = Nutrient treatment (green), W-N- = Water shortage treatment (red).

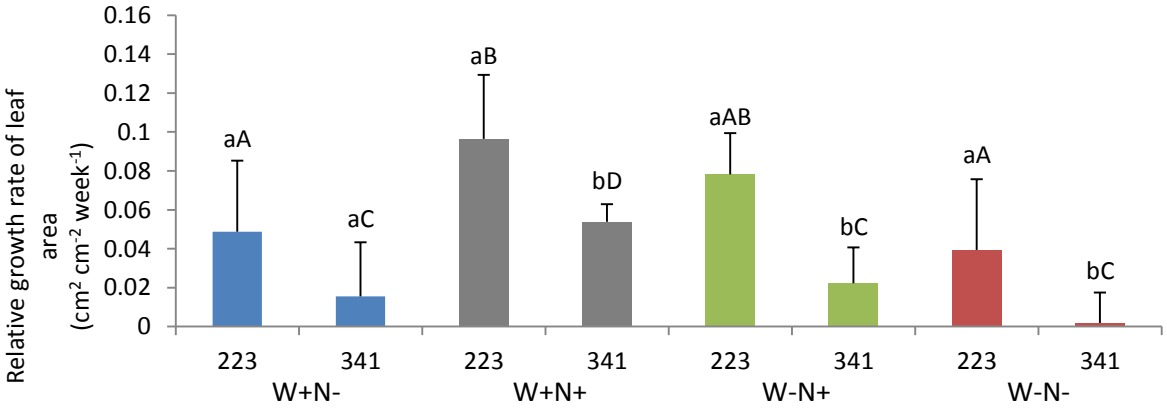

Figure 4. Relative growth rate of mean leaf area (cm^2 day^{-1}) of two willow genotypes (223 and 341), greenhouse grown in four experimental treatments. Significant differences between treatments, within genotype, are marked with different upper case letters (genotype 223: A-B; genotype 341: C-D) and significant differences between genotype, within treatment, are marked with lower case letters. Treatments: W+N- = Control (blue), W+N+ = Water and nutrient treatment (grey), W-N+ = Nutrient treatment (green), W-N- = Water shortage treatment (red).

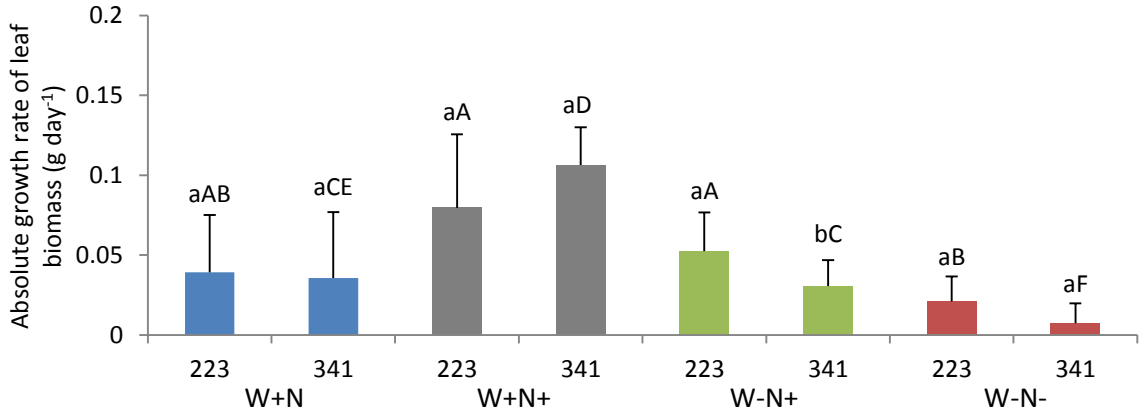

Figure 5. Absolute growth rate of mean leaf biomass (g day^{-1}) of two willow genotypes (223 and 341) greenhouse-grown in four experimental treatments. Significant differences between treatments, within genotype, are marked with different upper case letters (genotype 223: A-B; genotype 341: C-F) and significant differences between genotype, within treatment, are marked with lower case letters. Treatments: W+N- = Control (blue), W+N+ = Water and nutrient treatment (grey), W-N+ = Nutrient treatment (green), W-N- = Water shortage treatment (red).

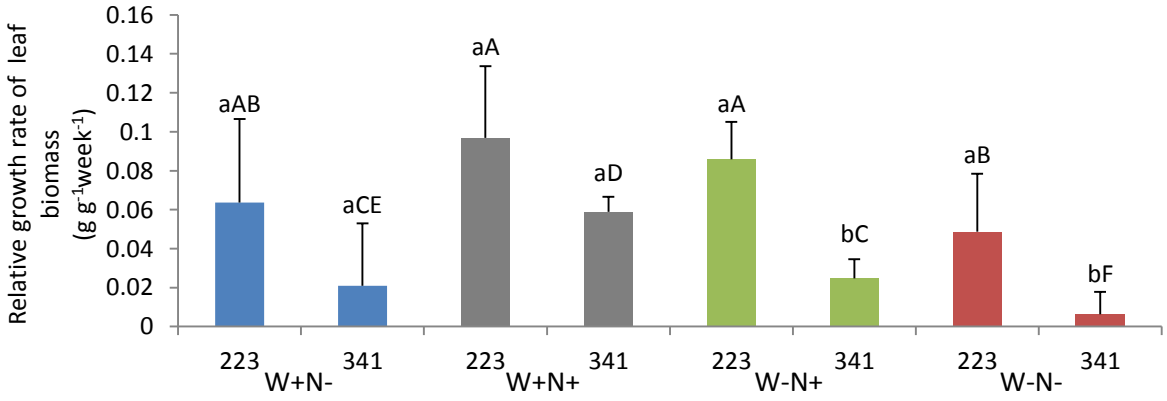

Figure 6. Relative growth rate of mean leaf biomass (g day^{-1}) of two willow genotypes (223 and 341) greenhouse-grown in four experimental treatments. Significant differences between treatments, within genotype, are marked with different upper case letters (genotype 223: A-B; genotype 341: C-F) and significant differences between genotype, within treatment, are marked with lower case letters. Treatments: W+N- = Control (blue), W+N+ = Water and nutrient treatment (grey), W-N+ = Nutrient treatment (green), W-N- = Water shortage treatment (red).

Plants of genotype 223 with nutrient treatment (W-N+) produced aboveground biomass significantly faster than genotype 341 according to RGR. On the contrary, plants of genotype 341 with water and nutrient treatment (W+N+) grew significantly faster than plants of genotype 223, according to absolute growth rate. No other significantly differences between the genotypes within the treatments for neither absolute growth rate nor RGR were observed (table 2; table 3). Plants of genotype 341 with water and nutrient treatment (W+N+) also produced aboveground biomass significantly faster than the other treatments within genotype 341, according to both absolute growth rate and RGR. Similar results were observed for absolute root growth rate of roots, growth were significantly faster for water and nutrient treated (W+N+) plants of genotype 341 comparing plants with nutrient treatment (W-N+) (table 2; figure 7). Though, growth rate was not significantly faster than non-fertilized plants (W+N-; W-N-) (table 2; figure 7).

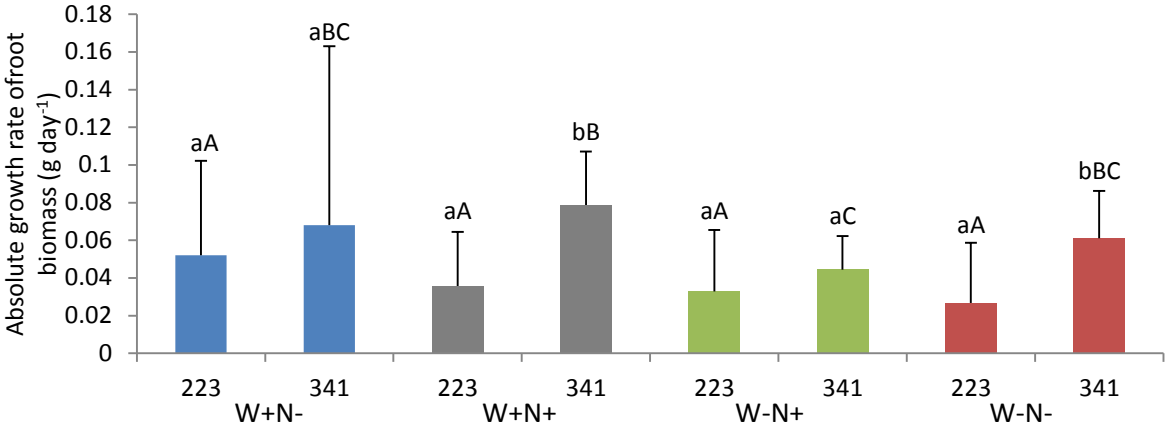

Figure 7. Absolute growth rate of mean root biomass (g day^{-1})) of two willow genotypes (223 and 341) greenhouse-grown in four experimental treatments. Significant differences between treatments, within genotype, are marked with different upper case letters (genotype 223: A; genotype 341: B-C) and significant differences between genotype, within treatment, are marked with lower case letters. Treatments: W+N- = Control, W+N+ = Water and nutrient treatment, W-N+ = Nutrient treatment, W-N- = Water shortage treatment.

6.1.2 Above-, belowground biomass and morphological traits

Production of aboveground biomass, size of stem diameter, leaf biomass and leaf area was significantly affected by the genotype (table 4). Plants of genotype 341 with water and nutrient treatment (W+N+) and with water shortage treatment (W-N-) produced significantly more above ground biomass (6.7 g respectively 2.6 g) (figure 8), root biomass (1.67 g respectively 1.29 g) (figure 9) and leaf area (926 cm^2 respectively 320 cm^2) (figure 10) compared to plants of genotype 223.

Table 4. ANOVA table of F-values and P-values of aboveground biomass, stem diameter and leaf area (for the both genotypes (223 and 341) and all four treatments (control; water and nutrients; nutrients; water shortage))

*** = p<0.001, ** = p<0.01, * = p<0.05, n.s. = p>0.05

Variable	Genotype		Treatment		Genotype : Treatment		Block	
	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value
Aboveground biomass (g)	15.61	***	11.95	***	1.85	n.s.	0.02	n.s.
Stem diameter (cm)	6.40	*	2.54	n.s.	0.34	n.s.	0.47	n.s.
Leaf area (cm^2)	23.70	***	20.75	***	1.12	n.s.	1.55	n.s.

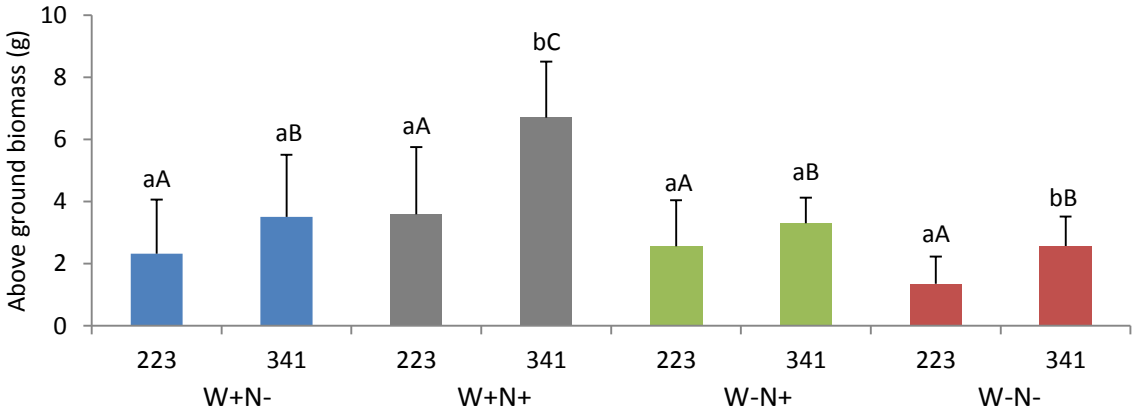

Figure 8. Mean aboveground biomass (g) of two willow genotypes (223 and 341) greenhouse-grown in four experimental treatments. Significant differences between treatments, within genotype, are marked with different upper case letters (genotype 223: A; genotype 341: B-C) and significant differences between genotype, within treatment, are marked with lower case letters. Treatments: W+N- = Control (blue), W+N+ = Water and nutrient treatment (grey), W-N+ = Nutrient treatment (green), W-N- = Water shortage treatment (red).

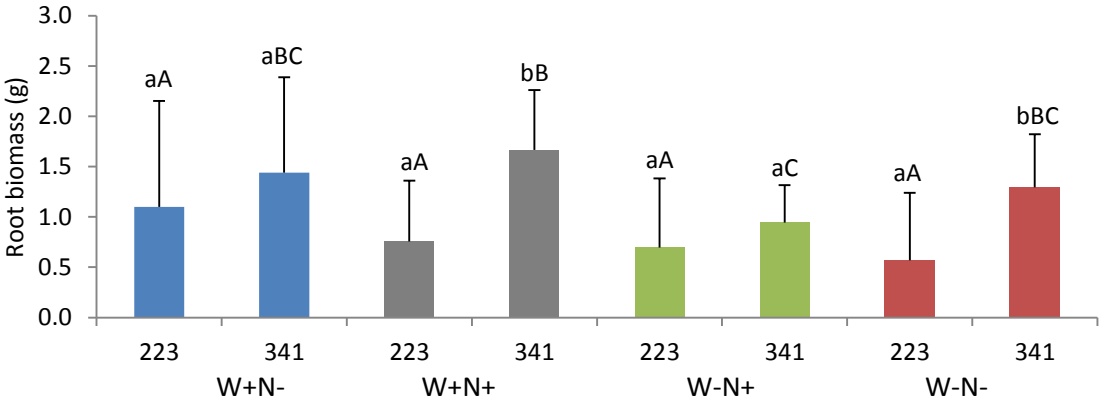

Figure 9. Mean root biomass (g) of two willow genotypes (223 and 341) greenhouse-grown in four experimental treatments. Significant differences between treatments, within genotype, are marked with different upper case letters (genotype 223: A-B; genotype 341: C-D) and significant differences between genotype, within treatment, are marked with lower case letters. Treatments: W+N- = Control (blue), W+N+ = Water and nutrient treatment (grey), W-N+ = Nutrient treatment (green), W-N- = Water shortage treatment (red).

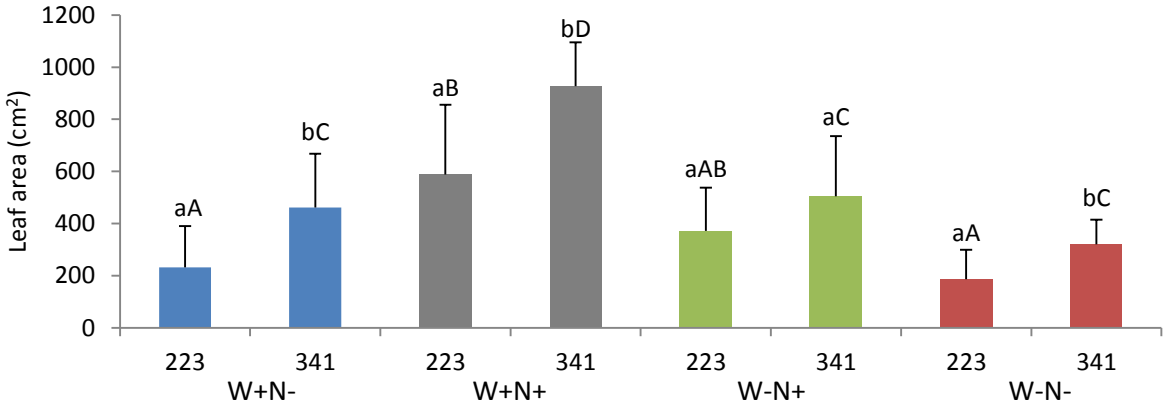

Figure 10. Mean leaf area (cm^2) of two willow genotypes (223 and 341) greenhouse-grown in four experimental treatments. Significant differences between treatments, within genotype, are marked with different upper case letters (genotype 223: A-B; genotype 341: C-D) and significant differences between genotype, within treatment, are marked with lower case letters. Treatments: W+N- = Control (blue), W+N+ = Water and nutrient treatment (grey), W-N+ = Nutrient treatment (green), W-N- = Water shortage treatment (red).

Plants of genotype 341 with water and nutrient treatment (W+N+) produced the greatest amount of aboveground biomass, leaf biomass and leaf area. Similar, fertilized plants (W+N+; W-N+) of genotype 223 produced greater leaf area and leaf biomass comparing the other treatments within genotype 223 (figure 10; figure 11). Production of aboveground biomass and root biomass of genotype 223 were on the other hand not significantly affected by the treatments (figure 8; figure 9). Additionally plants of genotype 341 with water shortage treatment (W-N-) produced significantly more leaf biomass (figure 11) (1.06 g) than genotype 223 (0.64 g) and plants of genotype 341 visually produced wider stems than plants of genotype 223.

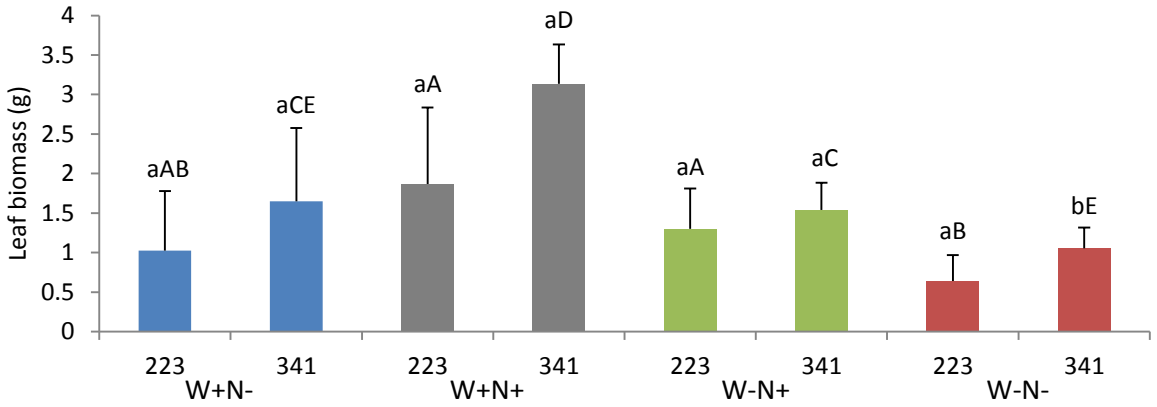

Figure 11. Mean leaf biomass (g) of two willow genotypes (G223 and G341) greenhouse-grown in four experimental treatments. Significant differences between treatments, within genotype, are marked with different upper case letters (genotype 223: A-B; genotype 341: C-D) and significant differences between genotype, within treatment, are marked with lower case letters. Treatments: W+N- = Control (blue), W+N+ = Water and nutrient treatment (grey), W-N+ = Nutrient treatment (green), W-N- = Water shortage treatment (red).

Water shortage plants (W-N-) of genotype 341 produced significantly more root biomass in relation to shoot biomass compared with the other treatments within genotype 341 (figure 12). Water and nutrient treated plants (W+N+) and water shortage treated plants (W-N-) of genotype 341 produced significantly more root biomass in relation to shoot biomass compared with plants of genotype 223 (table 6; figure 12). The relationship were inversely for plants with control treatment (W+N-) and with nutrient treatment (W-N+), though without significance differences between the genotypes (table 7; figure 12). Nutrient treated plants (W-N+) of genotype 223 produced significantly longer roots (231 m g^{-1}) than plants of genotype 341 (175 m g^{-1}) (table 6; table 5). No other significant observations were made regarding SRA, SRL (table 6; table 7) or stem height.

Table 5. Absolute values of specific root area (SRA) and specific root length (SRL) for roots of two genotypes (223 and 341) with four treatments (W+N- = Control, W+N+ = Water and nutrient treatment, W-N+ = Nutrient treatment, W-N- = Water shortage treatment).

	SRA m^2 g^{-1}		SRL m g^{-1}	
	223	**341**	**223**	**341**
W+N-	0.17	0.16	267	257
W+N+	0.25	0.17	322	237
W-N+	0.17	0.13	231	175
W-N-	0.17	0.13	265	190

Table 6. Wilcoxon table of p-values of differences in leaf biomass, root biomass, shoot to root ratio, specific root area and specific root length between genotypes (223 and 341) within treatments (W+N- = Control, W+N+ = Water and nutrient treatment, W-N+ = Nutrient treatment, W-N- = Water shortage treatment). Differences between genotypes are marked with:

** = p<0.01, * = p<0.05, n.s. = p>0.05

Treatment	Leaf biomass (g) Between genotypes	Root biomass (g)	Shoot : Root	SRA	SRL
W+N-	n.s.	n.s.	n.s.	n.s.	n.s.
W+N+	**	*	*	n.s.	n.s.
W-N+	n.s.	n.s.	n.s.	n.s.	*
W-N-	*	*	*	n.s.	n.s.

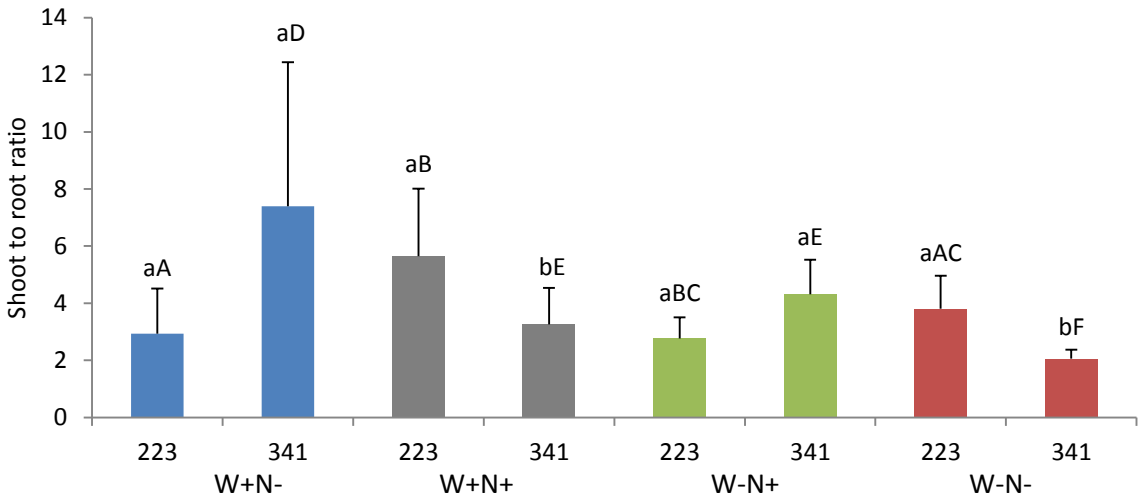

Figure 12. Mean root to shoot ratio of two willow genotypes (G223 and G341) greenhouse-grown in four experimental treatments. Significant differences between treatments, within genotype, are marked with different upper case letters (genotype 223: A-B; genotype 341: C-D) and significant differences between genotype, within treatment, are marked with lower case letters. Treatments: W+N- = Control (blue), W+N+ = Water and nutrient treatment (grey), W-N+ = Nutrient treatment (green), W-N- = Water shortage treatment (red).Morphological results

Table 7. Wilcoxon table of p-values of differences in leaf biomass, root biomass, shoot to root ratio, specific root area and specific root length between treatments (W+N- = Control, W+N+ = Water and nutrient treatment, W-N+ = Nutrient treatment, W-N- = Water shortage treatment) within genotypes (223 and 341). Differences between genotypes are marked with:

** = p<0.01, * = p<0.05, n.s. = p>0.05

	Leaves (g)		Roots (g)		Shoot : Root		SRA		SRL	
	Within genotype									
Treatment	223	341	223	341	223	341	223	341	223	341
W+N- & W+N+	n.s.	***	n.s.	n.s.	*	**	n.s.	n.s.	n.s.	n.s.
W+N- & W-N+	n.s.	n.s.	n.s.	n.s.	**	*	n.s.	n.s.	n.s.	n.s.
W+N- & W-N-	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.
W+N+ & W-N+	n.s.	***	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
W+N+ & W-N-	*	***	n.s.	n.s.	**	***	n.s.	n.s.	n.s.	n.s.
W-N+ & W-N-	**	**	n.s.	n.s.	n.s.	***	n.s.	n.s.	n.s.	n.s.

Nineteen of the replicates had some abscised leaves by time of harvest (5 with water and nutrient treatment; 8 with nutrient treatment; 6 with water shortage treatment). Sixteen of nineteen were of genotype 341, and the three of genotype 223 had nutrient treatment (2 plants) and water shortage treatment (1 plant). Abscised leaves are not included in the analysis.

6.1.3 Nitrogen content and nitrogen concentration in leaves

Fertilized plants had a higher N content compared with unfertilized plants (figure 13) and lowest N content was observed in the bottom of the plant (table 8). The N content and the N concentration depended on genotype and nutrient availability (table 8; table 9), additionally did water availability impact N concentration (table 9). Plants of genotype 223 with water and nutrient treatment (W+N+) and plants with water shortage treatment (W-N-) had a significantly higher N content than plants of genotype 341 (figure 13), however no significantly differences in N concentration were observed. A higher N content or greater N concentration could not be observed due to drought. Though, N content in the bottom of drought suffered plants (W-N+; W-N-) showed a positive correlation with aboveground biomass (R = 0.85), the correlation between the two parameters declined in the middle and the top of the plant. Additionally, genotype 223 showed to contain more N in relation to absolute growth rate compared with genotype 341.

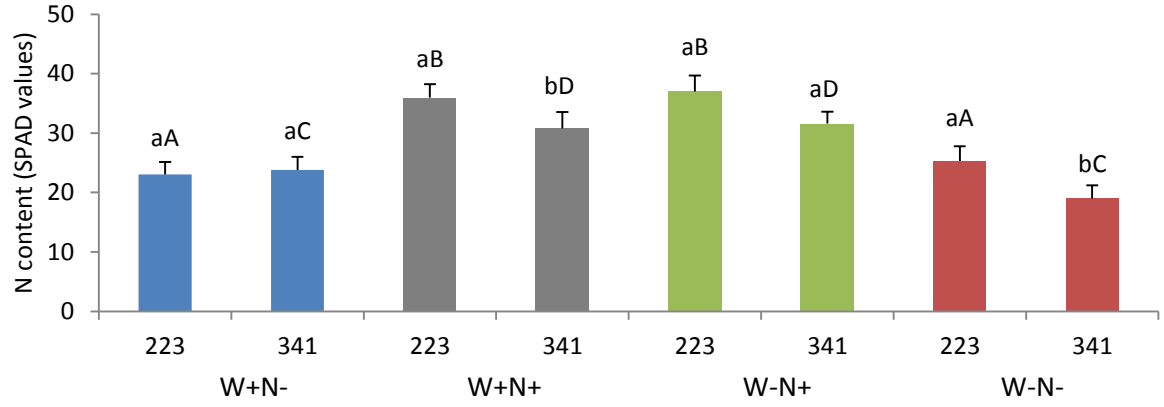

Figure 13. Significant differences of mean leaf N content for the whole plant between treatments, within genotype, are marked with different upper case letters (genotype 223: A-B; genotype 341: C-D) and significant differences between genotype, within treatment, are marked with lower case letters. Treatments: W+N- = Control, W+N+ = Water and nutrient treatment, W-N+ = Nutrient treatment, W-N- = Water shortage treatment.

Table 8. ANOVA table for N content (SPAD values) in leaves at three heights (bottom, middle and top) at all willow plants (genotype 223 and 341 with four treatments; control, water and nutrient, nutrient, water shortage) used in the greenhouse experiment at final harvest.
.*** = p<0.001, ** = p<0.01, * = p<0.05, n.s. = p>0.05

	Top of plant		Middle of plant		Bottom of plant	
	F value	P value	F value	P value	F value	P value
Genotype	1.47	n.s.	26.50	***	8.27	**
Treatment	43.62	***	18.25	***	14.81	***
Treatment:Genotype	0.68	n.s.	2.82	n.s.	2.17	n.s.
Block	0.18	n.s.	1.11	n.s.	0.83	n.s.

Table 9. ANOVA table for N concentration at final harvest of all plants (genotype 223 and 341 with four treatments; control, water and nutrient, nutrient, water shortage) used in the greenhouse experiment.
*** = p<0.001, ** = p<0.01, * = p<0.05, n.s. = p>0.05

	N concentration	
	F-value	**P-value**
Block	0.81	n.s.
Nitrogen	5.10	*
Water	5.15	*
Genotype	12.61	**
Nitrogen:Genotype	1.97	n.s.
Water:Genotype	1.97	n.s.

6.1.4 Soil moisture of pots

Soil of plants receiving less water (250 ml week^{-1}) had lower soil moisture than soils receiving water more often (3*250 ml week^{-1}) (figure 14). From figure 8 it can be deduced that soil in pots with plants of genotype 223 had lower soil moisture than soil in pots with genotype 341. Plants of genotype 223 grown in pots where soil moisture was measured produced less aboveground biomass than genotype 341 (figure 15).

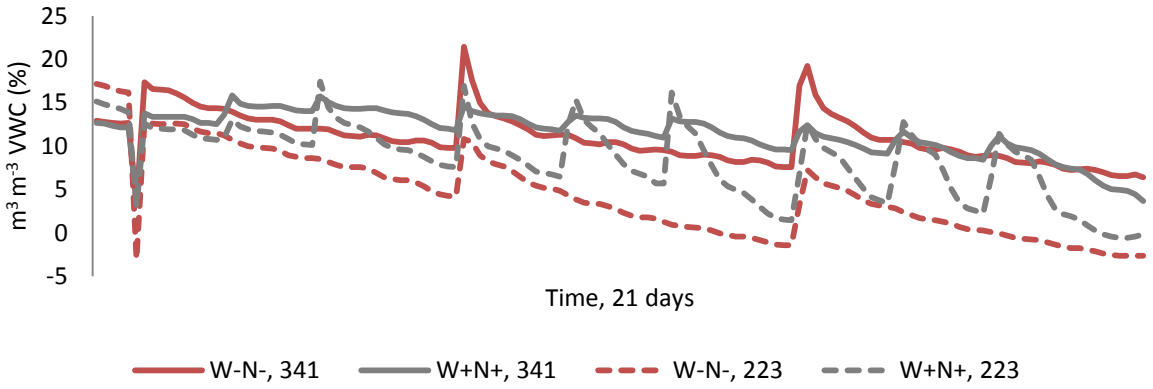

Figure 14. Soil moisture (%) measured in one pot per treatment (W+N+; W-N-) and genotype (223 and 341) (Treatments: W-N- = water shortage treatment (red) and W+N+ = water and nutrient treatment (grey)). Broken likes show soil moisture in pots with plants of genotype 223 and unbroken lines show soil moisture in pots with plants of genotype 341.

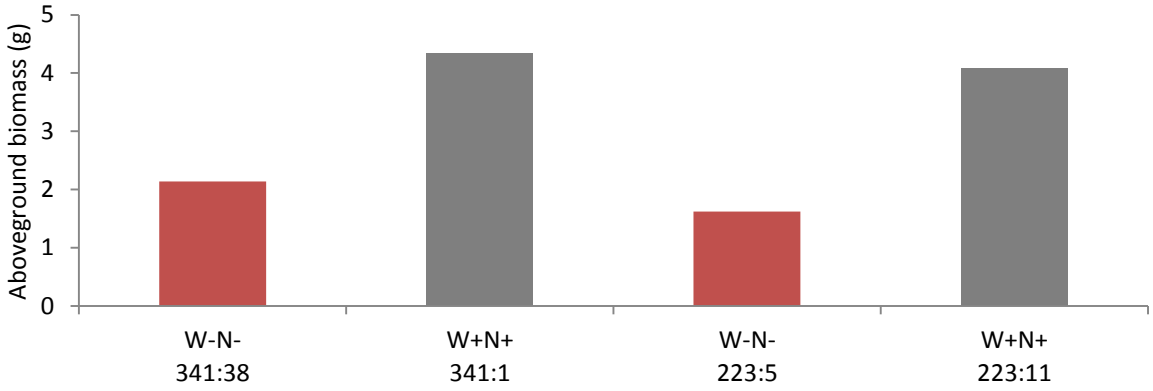

Figure 15. Aboveground biomass (g) of the plants grown in pots where soil moisture was measured. Two plants per genotype with respectively two different treatments (Treatments: W-N- = water shortage treatment (red) and W+N+ = water and nutrient treatment (grey)).

6.2 Field experiment

Vessel area were significantly affected by water availability (table 10), with a visually greater vessel area for irrigated plants (figure 16) and significantly greater vessel area for irrigated plants of genotype 223 (figure 16). In contrary, genotype 341 produced significantly greater total vessel area (228 mm^2 $stem^{-1}$) compared to genotype 223 (102 mm^2 $stem^{-1}$) (table 10), with the greatest area for irrigated plants (237 mm^2). Whereas genotype 223 produced the greatest area for non-irrigated plants (88 mm^2 $stem^{-1}$). Though, no significant observations were made between the treatments within respective genotype.

Table 10. ANOVA table of measured variables (amounts of vessels, mean vessel area, total vessel area, stem diameter, annual ring 1-3) of stems from Italy. Treatments: irrigated and non-irrigated. Genotypes: 223 and 341.

*** = p<0.001, ** = p<0.01, * = p<0.05, n.s. = p>0.05

	Treatment		Genotype		Treatment : Genotype	
Variable	**F-value**	**P-value**	**F-value**	**P-value**	**F-value**	**P-value**
Amounts of vessels	0.62	n.s.	5.10	n.s.	0.06	n.s.
Mean vessel area	25.84	***	2.72	n.s.	1.71	n.s.
Total vessel area	0.01	n.s.	6.14	*	0.22	n.s.
Stem diameter	1.45	n.s.	5.23	n.s.	0.12	n.s.
Annual ring 1	0.40	n.s.	0.34	n.s.	0.64	n.s.
Annual ring 2	0.78	n.s.	4.95	n.s.	0.00	n.s.
Annual ring 3	1.48	n.s.	3.31	n.s.	0.02	n.s.

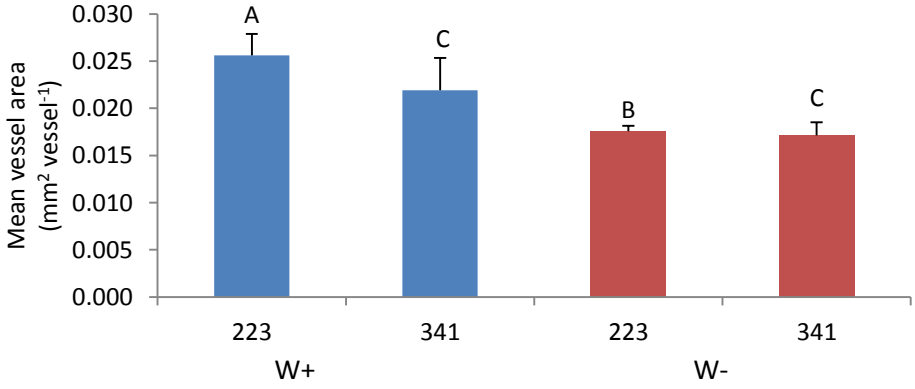

Figure 16. Column diagram of mean vessel area (mm^2) for different treatments (irrigated = W+ (blue); non-irrigated = W- (red)). Significant differences between genotypes with uppercase letters (genotype 223: A-B; genotype 341: C).

Genotype 341 produced wider stem diameter compared to genotype 223 (p = 0.0515) and irrigation did not seem to have an on impact stem diameter (table 10). Thought, stem diameter showed to be positive correlated with total vessel area (R = 0.93) (figure 17A) and amounts of vessels (R = 0.96) (figure 17B).

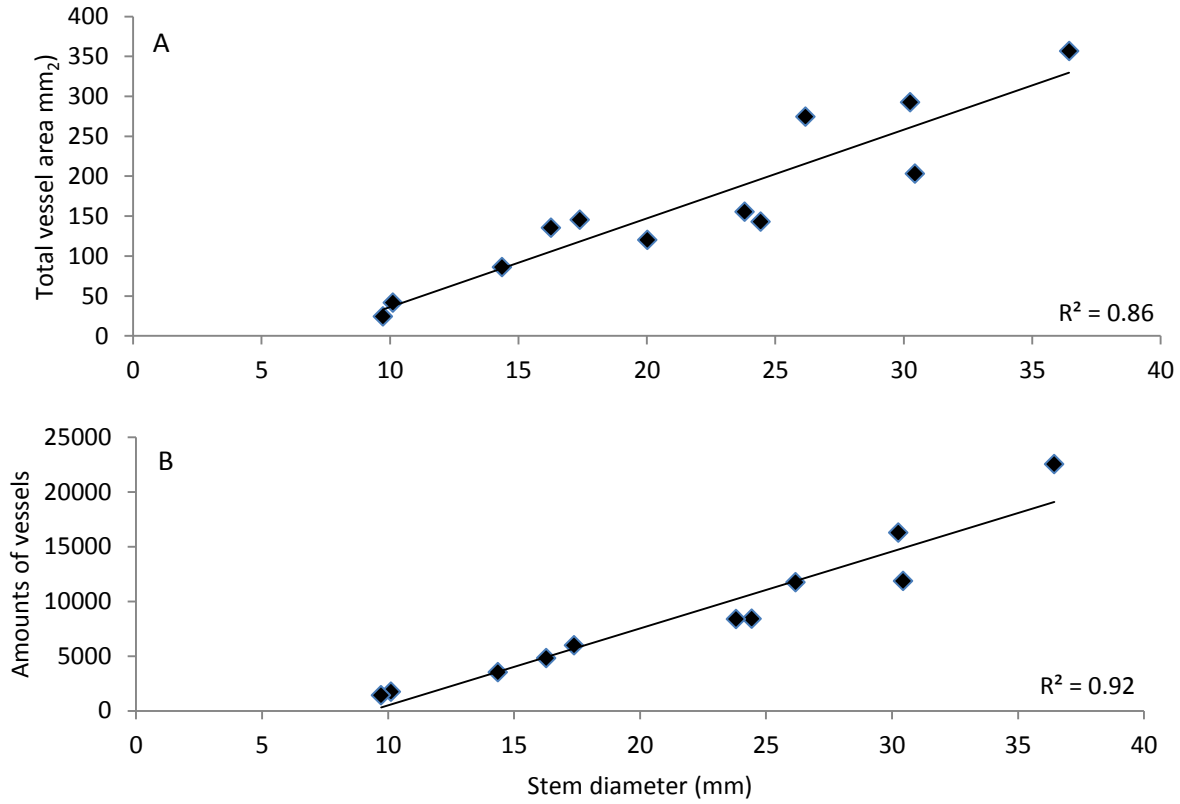

Figure 17. Scatter plot of stem diameter and total vessel area per stem (mm^2) (A) and amounts of vessels (B) for two genotypes (223 and 341) and two treatments (irrigated and non-irrigated). Data collected from plants grown in field in Italy for three years. Stem diameter: y = 11.105x - 75.087, R^2 = 0.86). Amounts of vessels: y = 703.55x – 6525, R^2 = 0.92.

Correlation between amounts of vessels and mean vessel area is plotted in a scatterplot diagram (figure 18), showing a negative correlation of the fewer vessels the greater area. The low R-value (R= 0.51) indicates poor correlation between the two parameters.

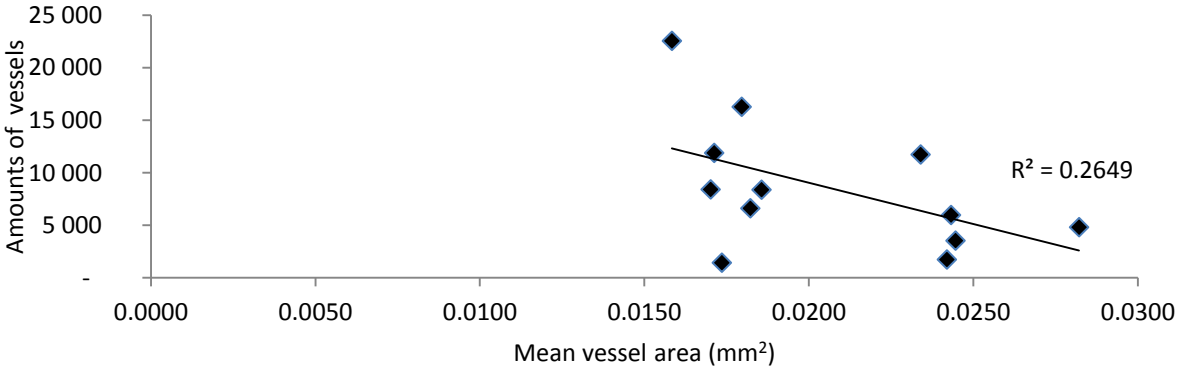

Figure 18. Scatter plot of mean amount of vessels and mean vessel area, y = -17169ln(x) – 58386 (R^2 = 0.28), for two genotypes (223 and 341) and two treatments (irrigated and non-irrigated).

A negative linear correlation between amounts of vessels and $\delta^{13}C$ (R = 0.77) respectively standardized WUE (R = 0.79) is shown in figure 19. The lower content of $\delta^{13}C$ in correlation with a greater amounts of vessels (figure 19A) is related to the standardized WUE (figure 19B), due to a lower WUE indicates a higher water use and higher range or longer period of open stomata and thereby a lower uptake of ^{13}C. All though no true conclusions can be made,

due to few replicates (n=2 per genotype; treatment: W-). Additionally, genotype 223 had fewer amounts of vessels compared to stems of genotype 341.

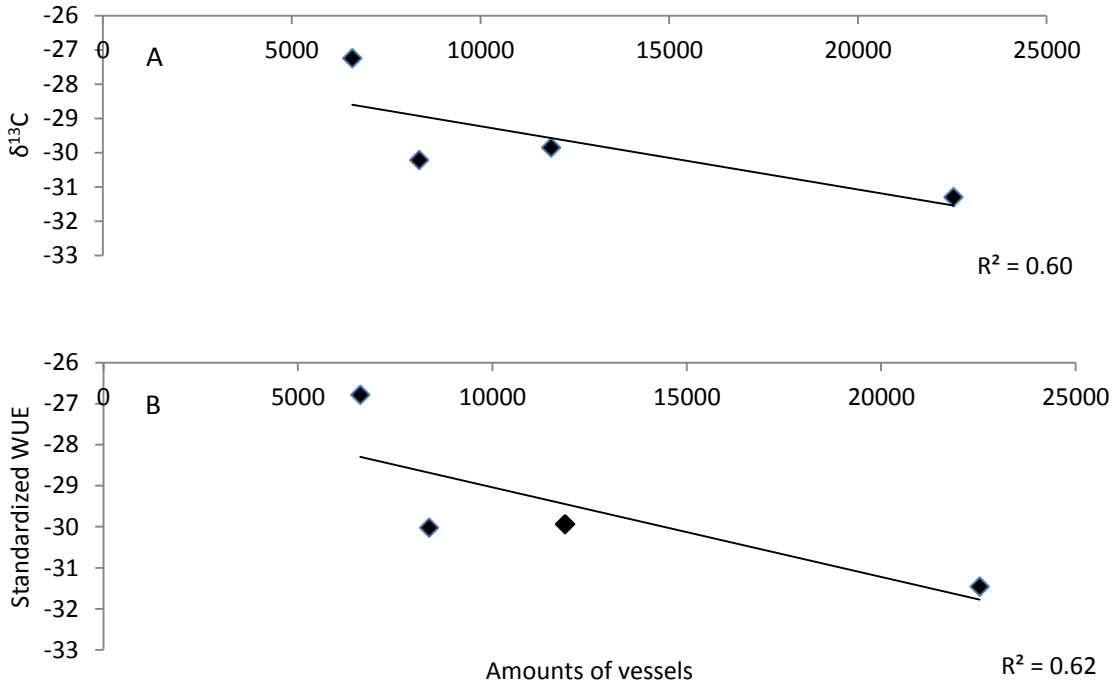

Figure 19. Scatter plot of mean amounts of vessels and δ13C (y = -0.0002x - 27.375; R^2 = 0.60) (A) and standardized water use efficiency (y = -0.0002x - 26.859; R^2 = 0.62) (B) for two genotypes (23 and 341) and non-irrigated plants. Data collected from plants grown in field in Italy for three years.

7 DISCUSSION

7.1 Greenhouse experiment

For this experiment, plants of genotype 341 with water and nutrient treatment (W+N+) produced the greatest aboveground biomass, leaf biomass and leaf area. All wanted traits for bioenergy production, thus the aboveground biomass of willow is used for the energy production. Irrigation of plants of genotype 341 increased growth of aboveground biomass and drought reduced biomass production, which is a well-known effect (Wikberg and Ögren, 2007). Drought suffered plants (W-N+; W-N-) of genotype 341 reduced its aboveground biomass with 27 % for non-fertilized plants (W-N- vs. W+N-) and with 51 % for fertilized plants (W+N+ vs. W-N+), indicating the impact of drought events for yield. In contrary, irrigation of genotype 223 did not significantly affect aboveground biomass production. Possibly due to an overall lower growth of genotype 223 during the experiment.Previous results also states insignificant effect of drought on some genotypes (Van Splunder et al., 1996).

Just like Silvola & Ahlholm (1992) this study shows that the nitrogen level in the soil is reflected in the plants. In opposite from other studies (Glynn et al., 2004; Weih et al., 2011) this study could not confirm that drought suffered plants have a greater N concentration than well-watered plants. A reason for this may be the short period of treatment. It is also stated that a limited water acquisition can lead to declined N uptake, even though there is available N (Corbeels et al., 1998). Neither this could be confirmed by this study, thus greatest N content was observed in plants receiving N, independent of availability of water.

Nutrient treated plants (W-N+) for genotype 223 produced significantly longer roots than genotype 341, but no other significant differences regarding SRL or SRA were shown. Though, I can confirm that the genotype plays important roles for root production and that low water conditions led to greater root biomass compared to well-watered conditions. For both irrigated and fertilized (W+N+) conditions and non-irrigated and non-fertilized (W-N-) conditions plants of genotype 341 produced significantly more root biomass than plants of genotype 223. Thereby, genotype 341 has bigger resources for taking up water and solved nutrients for producing more biomass (Lambers et al., 2006). The differences between the genotypes may also indicate that genotype 341 is better suited for rapid environmental changes due to the bigger root system.

Greenhouse experiments have some advantages compared to a field experiments. The trials are under relatively controlled conditions, for example regarding air humidity, temperature, irrigation (no precipitation) and fertilization (no additional due to droppings etc.). One advantage with this experiment was the possibilities to have four different treatments which symbolized different growing conditions in relation to water and nutrients. It was beneficial to be able to see the genotypes' reactions in the different treatments and also to see how the genotypes differed from each other within each treatment.

Another advantage with this study was the usage of already relatively known genotypes. Different amounts of biomass production of the two genotypes were expected, due to the different amounts of production, of the used genotypes, in the Italian field experiment (Beyer et al., unpubl.). This greenhouse experiment can therefor contribute with important results affecting future studies regarding these genotypes and conclude that genotype 341 is probably better suited for biomass production than genotype 223.

One of the limitations with the greenhouse experiment were the slow emerge of plants of genotype 223, leading to great differences in initial biomass production between the plants of genotype 223 and also a lower initial production of genotype 223 compared to genotype 341. Of course it would have been easier if all plants were in the same growth stage when treatment period started, but it can be hard to influence especially due to the genetic differences. When harvesting the plants and removing the cuttings from the soil it appeared that some of the shoots of genotype 223 grew from nodes in the bottom of the cutting. Consequently the shoots had to grow through the soil before reaching soil above soil surface. Plants of genotype 341 did not emerge in this way and the differences in emerging decreased growth of genotype 223 compared to genotype 341. This emerging trait of genotype 223 is unfavorable for a commercial genotype and from that perspective is genotype 223 not recommended to use for bioenergy production.

To compensate for the differences in initial production I calculated RGR for leaf area, stem height and leaf, stem, aboveground and root biomass to evaluate the differences growth between both the genotypes and to see how the treatments affected growth rate. Though, there are some problems with this equation generating in misleading information. Growth rate of plants tend to slow down with growth and therefore a low initial size will generate a greater RGR (Philipson et al., 2012). This is a possible reason for the greater RGR of genotype 223 compared to genotype 341, thus generally genotype 341 produced greater biomass throughout the experiment with a greater absolute growth rate. Additionally, results from the field trial in Italy (where stems to this study were collected) show that genotype 223 has a total lower biomass production after three years compared to genotype 341 (Beyer et al., unpubl.). This indicates that growth rate of genotype 223 is lower and thereby may the RGR calculations in this study be misleading.

For future greenhouse experiment similar to this I recommend to measure soil moisture in more pots than in only four. The result from this study only provides indications of differences between the genotypes and the treatments and it would be interesting to closer evaluate the water use of the plants. The pots where soil moisture was measured plants of genotype 223 had a lower soil moisture compared to the pots were plants of genotype 341 grew. Additionally, the plants of genotype 223 produced less aboveground biomass indicating that genotype 223 has a lower WUE according to explanations of WUE by Condon et al. (2002) and Slípková and Pokorný (2012). Though, as mentioned before these results are limited due to few replicates but shows an indication of the genotypes' water use and aboveground production.

Even though it is not unusual that older leaves of plants lead to abscission for example due to self-shading and translocation of nutrients to younger plant parts (Weih and Rönnberg-Wästljung, 2007) this phenomenon might affected the results of leaf and aboveground biomass negatively. Thus the treatment period only lasted for three weeks the loss of leaf biomass differed between the genotypes and thereby changed the proportion of living leaf biomass at harvest time compared to total produced biomass under time of growth. Most of the replicates with abscised leaves were of genotype 341, this in combination with the overall higher biomass production of genotype 341 indicates higher growth potential compared to genotype 223. Another reason for abscised leaves, except from the natural behavior, can be drought (Savage et al., 1999) or N deficit (Larsen 1998). N deficit is firstly shown on older

leaves, the leaves turns yellow and thereafter becomes necrotic (Larsen 1998) and have also seen yellow and necrotic leaves on plants in the greenhouse experiment.

Finally, a longer experiment is recommended for future studies. Due to low emerge and slow growth in the beginning of the experiment delayed start of treatment resulted in a shorter treatment period. A longer time before the start of the treatment would probably have increased the initial production, especially of plants of genotype 223 and decreased the initial production between the genotypes. A longer treatment period may have generated significant differences in N concentration between irrigated and non-irrigated plants and decreased differences between the genotypes in amounts of abscised leaves. It may also generate greater differences in both aboveground biomass production between the genotypes based on genotypes' biomass production in Italy (Beyer et al., unpubl.) and also in increased differences for root biomass production. However, longer treatment period does not always generate significant differences in root biomass production (Van Splunder et al., 1996). This study did not show any significant differences in neither SRL nor SRA due to low water conditions, something a longer treatment is expected to show. At least for root length, where both drought (Van Splunder et al., 1996; Rytter and Hansson, 1996) and N deficit (Ericsson, 1981) has shown to lead to longer roots. Another possible reason for insignificance differences between genotypes for above mention parameters may also, of course, be that there are no or few genotypic differences for these parameters and different responses between the genotypes are not to be expected.

Water and nutrient treatment (W+N+) is shown to significantly increase growth rate of aboveground biomass production for plants of genotype 341. Availability of nutrients played a significant role in absolute leaf area growth rate, for both genotypes and for RGR of genotype 223. This study can therefore state that N deficit can lower growth, like previously proven (Campbell et al., 2008; Ericsson 1981; Taylor, 1993; Tahvanainen, 1999; Cunningham et al., 1999). Though, something that should be taken in consideration is the great standard deviations for growth rate of leaf area, in spite of high values there are significant differences between treatments, indicating that growth beneficial treatments (high water availability and N fertilization) are important for high production.

Nutrients also positively impacted absolute growth rate of leaf biomass of genotype 341 but not RGR for neither one of the genotypes. Though, RGR of leaf biomass is significantly slower for water shortage plants (W-N-) of genotype 223 compared to fertilized plants (W+N+; W-N+) showing that drought slows down growth. On the other hand there are no significant differences in RGR of leaf biomass between control treated plants (W+N-) and water shortage plants (W-N-) nor between control treated plants (W+N-) and fertilized plants (W+N+; W-N+).

This study shows that the genotype plays an important role for absolute root growth rate during favorable (W+N+) or water and nutrient limiting conditions (W-N-), genotype 341 produced root biomass faster than genotype 223. Choice of genotype can thereby be determining for final biomass production. On contrary, genotype is not a significant factor for root growth rate during good or bad conditions (W+N-; W-N+). Like other studies (Glynn et al., 2004; Van Splunder et al., 1996; Lambers et al., 2006) this study shows a proportionally increased root production due to drought. Though only for water shortage treated plants (W-N-)

of genotype 341. The N effect of nutrient treated plants (W-N+) may have increased aboveground biomass production and thereby increased the shoot to root ratio.

The only significant differences in root biomass production (within the genotypes) are for plants of genotype 341 between water and nutrient treatment (W+N+) and nutrient treatment (W-N+), indicating that available water increase root biomass. Possibly due to that plants with water and nutrient treatment (W+N+) produced more coarse roots in relation to fine roots, and thereby increases root weight more than for nutrient treated plants (W-N+) (Ryan et al., 1995). The greater root system of plants of genotype 341 with water and nutrient treatment (W+N+) and water shortage treatment (W-N-) compared to the root system of genotype 223. A greater root system facilitates water and nutrient uptake (Campbell et al., 2008) because of its greater nutrient uptake area compared to a smaller root system (Lambers, 2006) and the root system can thereby continue to grow even bigger. The greater root system of genotype 341 is reflected in the greater production of aboveground biomass compared to genotype 223, indicating that the greater root system simplifies water and nutrient uptake and biomass production.

N content in immature leaves depends on the development stage, cell division or cell elongation. During cell division increases biomass, through synthesis of high N/C cytoplasmic material. Cell elongation is driven by water and ion uptake in combination of increasing cell walls (Roggatz et al., 1999). N content may thereby differ between the leaves depending on where it is in the cycle. This is a reason for insignificant results between N content and the treatments respectively the genotypes for the upper and immature leaves. N content measurements are therefore recommended to mature leaves for a representative result.

Plants of genotype 223 has a visually greater N concentration and N content than plants of genotype 341, thus the results are not significant it is uncertain to make conclusions. Though, it might be related to that drought suffered plants have shown to increase its N concentration (Glynn et al., 2004; Weih et al., 2011) because the results from this study indicates that genotype 223 suffer more from drought than genotype 341.

7.2 Field experiment

Mean vessel area was significantly greater for irrigated plants of genotype 223 and visually greater for irrigated plants of genotype 341, showing that environmental factors affect the size of the vessels and thereby affect the water transport in the plants. Total vessel area and the amounts of vessels, on the other hand, was insignificantly affected by the treatments (irrigated or non-irrigated), but rather affected by genotype. Plants of genotype 341 produced greater total vessel area than genotype 223 and tended to have greater total vessel area for irrigated plants. In contrary plants of genotype 223 produced greater total vessel area for non-irrigated plants. The differences between the genotypes indicate that it is important to breed for genotypes that can resist rapid temporal or seasonal changes in water availability as well as for genotypes adapted for different environments.

One advantage of this part of the study is that the analyzed stems are field grown and the plants may therefore look more similar to plants at commercially grown field compared to greenhouse grown plants.

The used X-ray method is fine scaled and can obtain 3D-pictures and thereby possibilities for analyzing for example vessel length or other vertical tissues. Even though only cross-

sections of the stems were used in this study, the collected data can be used for future studies. It would for example be interesting to analyze if length of vessel element differed between both the genotypes and irrigated versus non-irrigated conditions.

It worked well with the X-ray analysis, though a higher resolution would increase the reliability of the analysis. The lower resolution aggravated the analysis and may have led to misleading results, for example it was difficult to distinguish the different annual rings from each other.

A field experiment can be more challenging to control compared to a greenhouse experiment, due to weather related difficulties and a field experiment has thereby more uncontrolled influencing parameters. Weather can interfere with optimal growth and thereby change growth pattern (Worbes, 1999; Walker et al, 2015), this may have happened in this field trial and the unexpected variability in the data caused low statistical power regarding annual rings.

The field experiment had also problems with insects. The insects lowered the leaf area by eating leaf tissue. Herbivory generates in smaller photosynthetic area and risk of reduced photosynthetic capacity in remaining tissue (Zangerl et al., 2002) and thereby lower incorporated energy.

A limitation with the analysis of the stems from the field experiment was that the stems were too few for providing reliable results, though the results shows indications and can be reason for future studies. Choice of replicates were based on stem diameter, choosing the smallest, middle and largest from each genotype and treatment because the analyzed were about to focus on mean vessel area and not stem diameter nor annual rings. That is probably the reason for insignificant results regarding diameter and ring size related to soil water status and the results from this experiment does not agree with Worbes (1999) and Walker et al. (2015) stating that availability of water affects size of annual rings. Additionally only four replicates (n=2 genotype^{-1}) were included in the regression analysis of amounts of vessels and $\delta^{13}C$ respectively standardized WUE, due to lack of data (of $\delta^{13}C$ and standardized WUE), more replicates are needed to improve reliability.

Just as other studies have shown (Hilarie and Graves, 1998; Kulkarni et al., 2010; Plavcová and Hacke, 2012) the water availability influenced the vessels; non-irrigated plants had a smaller mean vessel area than irrigated plants. Mean vessel area influences water conduction because conduction capacity scales as diameter to the fourth power (Tyree and Zimmerman, 2002; Wilson and Jackson, 2006), this in turn affects the transpiration, carbon fixation and eventually growth (Poorter et al., 2010). Growth rate have shown to be positively related to vessel diameter (Hoeber et al., 2014). Due to greater mean vessel area of genotype 223, plants of genotype 223 might have a greater growth rate than genotype 341. This was also shown in RGR calculations from the greenhouse experiment. Thus there are some doubts regarding the RGR for genotype 223 the above mentioned relation between vessel diameter and growth rate may not be applicable for this study.

Genotype 223 both had smaller total vessel area and fewer amounts of vessels compared to genotype 341, because stem diameter were smaller. This may indicate a higher conductivity of plants of genotype 223 thus higher conductivity is offered by fewer larger vessels compared to many smaller vessels (Sperry et al., 2007), but a high density of smaller vessels in-

crease resistance against embolism (Ewers et al., 2007). The possibly higher conductivity of plants of genotype 223 can be correlated with the results from the soil moisture measurement in the greenhouse experiment, where plants of genotype 223 seemed to transpire more water than plants of genotype 341, and were thereby less water use efficient.

Total vessel area and amounts of vessels showed to be positive correlated with stem diameter, with a greater stem diameter for genotype 341. Greater stem diameter for genotype 341 was also shown by Beyer at al. (unpubl.) Previous studies (Seguara, 2006; Brown, 1997) have shown positive correlations between stem diameter and aboveground biomass production, based on their results total vessel area and the amounts of vessels may be positive correlated with aboveground biomass production as well. If this theory is applicable, data for aboveground biomass can be calculated based on either amounts of vessels, total vessel area or possibly even standardized WUE or $\delta^{13}C$. This because the amounts of vessels were correlated with both $\delta^{13}C$ and standardized WUE. Growth rate have shown to be negatively related with wood density and amounts of vessel respectively $\delta^{13}C$ (Hoeber et al., 2014) and thus the greenhouse experiment showed a lower RGR for genotype 341 and the field experiment showed a greater amounts of vessels and lower values of $\delta^{13}C$ the observations of Hoeber et al. (2014) may be applicable to the results of this study.

8 CONCLUSIONS

Aboveground biomass productions of plants of genotype 341 were negatively affected by drought. Plants of genotype 223 were in contrary not significantly affected, though they produced significantly less aboveground biomass compared to genotype 341 (for water and nutrient treated (W+N+) and water shortage treated plants (W-N-)). Significantly slower absolute growth rate or RGR could not been observed due to drought and no changes in root morphology were measured.

However, plants of genotype 341 seem to have a greater drought tolerance than plants of genotype 223, especially under low nutrient conditions. Vessel area was negatively affected by drought conditions. Finally, fertilization increased growth and the combination of irrigation and fertilization increased aboveground biomass, leaf area and leaf biomass production significantly for plants of genotype 341.

To sum up; it is still unclear if genotype 223 or genotype 341 is the best genotype to grow for bioenergy production under dry and nutrient poor conditions. Even though genotype 341 produced the greatest amount of aboveground biomass, genotype 223 seem to have traits correlating to high relative growth rate and aboveground biomass production. Future studies within both morphology and anatomy are needed for deeper evaluations. Thus, analyzing the different anatomical traits of the xylem will contribute with further insight into the plant mechanism and level of adaptation to drought.

If expanding the vessel analyze it would be interesting look at the relationship between vessel area and annual rings and thereafter connect the results to climate data. It would also be interesting to take a closer look at and see how the vessel area changes depending on time of production.

For handling future energy questions and the development of bioenergy crops more research regarding willow and other SRC's is demanded. A tolerant and sustainable genotype is desired for growing. It is also important to develop genotypes for specific climates, to optimize production and secure energy resources.

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